The epidemiology of vaccine preventable virus infections: studies of rubella virus and hepatitis B virus infections performed by oral fluid testing

Thesis

THE EPIDEMIOLOGY OF VACCINE PREVENTABLE VIRUS INFECTIONS:

Studies of rubella virus and hepatitis B virus infections performed by oral fluid testing

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A thesis submitted in fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

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LIST OF ABBREVIATIONS

A	**adenosine**
Ag	**antigen**
anti-HBc	**antibody specific to hepatitis B core antigen**
BRHP	**Butajira rural health project**
C	**cytidine**
cDNA	**copy deoxyribonucleic acid**
CTF	**complement fixation test**
CRS	**congenital rubella syndrome**
DNA	**deoxyribonucleic acid**
dNTPs	**deoxynucleoside triphosphates**
E1	**rubella virus glycoprotein**
E2	**rubella virus glycoprotein**
ELISA	**enzyme linked immunosorbent assay**
EPI	**Expanded Programme of Immunisation**
FCS	**foetal calf serum**
FITC	**fluorescein isothiocyanate**
G	**guanosine**
GACELISA	**IgG antibody capture ELISA**
GACRIA	**IgG antibody capture radioimmunoassay**
GMT	**geometric mean titre**
GPV	**Global Programme of Vaccination**
HBcAg	**hepatitis B core antigen**
HBsAg	**hepatitis B surface antigen**

HBV	**hepatitis B virus**
HI	**haemagglutination inhibition**
HIV	**human immunodefficiency virus**
HRPO	**horse raddish peroxidase**
IDUs	**intravenous drug users**
IF	**immuno fluoresence**
IgA	**immunoglobulin A**
IgG	**immunoglobulin G**
IgM	**immunoglobulin M**
IU/ml	**international units per millilitre**
K	**kappa statistic**
LA	**latex agglutination**
MAB	**monoclonal antibody**
MACRIA	**IgM antibody capture radioimmunoassay**
mg/L	**milligrams per litre**
MMR	**measles, mumps and rubella**
NHS	**negative human sera**
NPA	**nasal pharyngeal aspirate**
NPV	**negative predictive value**
NRS	**normal rabbit serum**
nt	**nucleotide**
$OD_{450/620}$	**optical density at 450/620nm**
ORF	**open reading frame**
PBS	**phosphate buffered saline**
PCR	**polymerase chain reaction**

P/N	**positive control to negative control ratio**
PPV	**positive predictive value**
PSB	**pre-school boost**
r	**correlation coefficient**
RH	**radial haemolysis**
rHBcAg	**recombinant hepatitis B core antigen**
RNA	**ribonucleic acid**
RNase	**ribonuclease**
RIA	**radioimmunoassay**
ROC	**receiver operator characteristics**
RT-PCR	**reverse transcription PCR**
RV	**rubella virus**
T/N	**test sample to negative control ratio**
TMB	**tetra methyl benzidine**
TS	**throat swab**
U	**uracil**
ul	**microlitre**
WHO	**World Health Organisation**

Publications arising from work carried out in this thesis.

At the time of submission of this thesis four papers had been submitted and accepted for publication in the scientific literature. These are listed below and are included at the back of this thesis.

- **Vyse AJ**, Cohen BJ, Ramsay ME. A comparison of oral fluid collection devices for use in the surveillance of virus diseases in children. Public Health. 2001; 115:201-207.
- **Vyse A**, Cohen B. In saliva veritas. Communicable Disease and Public Health. 1999; 2: 298

- **Vyse AJ**, Brown DWG, Cohen BJ, Samuel R, Nokes DJ. Detection of rubella virus specific immunoglobulin G in saliva by an amplification based enzyme linked immunosorbent assay using monoclonal antibody to fluorescein isothiocyanate. Journal of Clinical Microbiology. 1998; 37:391-395.
- Nokes DJ, Enquselassie F, **Vyse A**, Nigatu W, Cutts FT, Brown DW. An evaluation of oral fluid collection devices for the determination of rubella antibody status in a rural Ethiopian community. Transactions of the Royal Society of Tropical medicine and Hygiene. 1998; 92:679-85.

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ABSTRACT

This thesis presents studies on the epidemiology of vaccine preventable virus infections investigated by oral fluid testing. It involved studies of both rubella and hepatitis B virus infections by the detection of specific IgG in oral fluid and of rubella virus by PCR detection and genotyping of viral nucleic acid. To select an oral fluid collection device with optimal performance for these studies three (Orasure, Omni-SAL, Oracol) were compared. Each device collected oral fluid of sufficient quality for qualitative analysis of virus specific IgG, but Oracol was most acceptable to the subjects being tested. IgG capture ELISA (GACELISA) tests were developed for detection of rubella and anti-HBc specific IgG in oral fluid. Their sensitivity was less than that of corresponding serum ELISAs, with sensitivity decreasing with increasing age of subjects. Whilst the performance of the rubella GACELISA was shown to be an improvement over the existing radioimmunoassay and was particularly sensitive with samples from paediatric populations, the performance of the anti-HBc GACELISA was not considered good enough for further use. The development of an RT-PCR assay targeting the E1 gene of rubella virus enabled the molecular epidemiology of rubella to be investigated using samples from the UK, China, Greece, and Brazil. By comparison to previously reported strains, the majority of strains were assigned to three banches of a phylogenetic tree. Those assigned to branches 1 (UK, Greece, China) and 2 (China) were not closely related to any previously reported strains. The timing of oral fluid collection and subsequent storage was critical for PCR detection. Ideally oral fluid samples should be collected within 14 days of the onset of symptoms and stored at least at -20^{0}C prior to testing.

INTRODUCTION

Vaccination is one of the most cost-effective measures that modern medicine has to offer for preventing infectious diseases that represent important causes of morbidity and mortality (90). When used appropriately and with a suitable epidemiological strategy, vaccines have had great success. Vaccination was responsible for the eradication of smallpox on a global scale, poliomyelitis from the Western hemisphere and under the guidance of the WHO Expanded Program on Immunization (EPI) has greatly reduced the incidence of several childhood infections including measles, diphtheria, tetanus and pertussis (97).

The word "immunity" comes from the Latin "*immunitas*" meaning exemption from public services, burdens or charges. It has now become associated with protection from infection and study of immunity forms a branch of the science of immunology (50). The concept of immunisation, vaccination and its ability to protect the public from infectious disease was pioneered by Edward Jenner in the late 18th century. His work was inspired by smallpox which, at this time, was endemic and often assumed epidemic proportions. It was a disease that was greatly feared as it was often fatal and left characterisitc disfiguring scarring from lesions in surviving cases.

Edward Jenner essentially carried out a study that experimentally tested the concept of vaccination against smallpox. Jenner had noted that persons infected with cowpox could not become infected with smallpox. He concluded that cowpox not only protected against smallpox but could also be transmitted from person to person as a "deliberate mechanism of protection" and therefore set out to investigate this. In May 1796 Jenner examined a dairy maid with fresh cowpox lesions on her finger and using

matter from these lesions inoculated an eight-year-old boy, James Phipps. Several weeks later he inoculated the boy again but this time with smallpox matter. No disease developed demonstrating complete protection. The Latin word for cow is "*vacca*" and cowpox "*vaccinia*", hence Jenner's procedure was named "vaccination" (50, 191).

Initially Jenner's work met with some skepticism and a paper describing his results was refused by the Royal Society. The reaction of the public was also not immediately favorable, early volunteer studies being unsuccessful. However, vaccination eventually became popular in London and "The Vaccination Act" of 1853 laid down that all infants must be vaccinated, though had no powers of enforcement whilst an earlier Act in 1840 introduced free vaccination following a three year epidemic of smallpox. These measures produced a marked decrease in the annual mortality from smallpox after only a few years and later vaccination acts saw the introduction of public vaccinators and compulsory vaccination. Compulsory vaccination was repealed in 1946 by the NHS Act and on 8th May, 1980, the World Health Assembly declared that smallpox was eradicated worldwide (191).

For nearly a century Jenner's smallpox vaccine was the only vaccine known until, during the 1880s, Louis Pasteur developed both an anthrax and a rabies vaccine. This led to a wide extension of immunizing methods against many diseases with correspondingly widened meanings of the terms "vaccination" and "vaccine", applying them to the use of both living and dead material. By the start of the First World War, typhoid vaccines were available and in 1921 Calmette and Geurin produced the BCG vaccine against tuberculosis, the first live bacterial vaccine for general human use (50).

Since the work of Pasteur in the late nineteenth century little development in viral vaccines occurred until, in 1930, Theiler announced a safe and effective vaccine against yellow fever which was further refined in 1937. Large scale production was carried out using embryonated hens eggs. The use of chick embryos to produce vaccine paved the way for production of other vaccines, particularly viral vaccines, on a large scale including a killed influenza vaccine produced in the early 1940s. A further technological advance important for vaccine development and large scale production was the establishment of cell culture methods for virus propagation. In the 1940s and 1950s poliomyelitis vaccines were made, and measles, rubella and mumps vaccines were pioneered in the 1950s and 1960s (135).

Enders, Weller and Robbins made a major technical advance in 1949 by demonstrating that, contrary to popular belief, poliomyelitis virus could be grown outside the primate central nervous system so enabling effective poliovaccines to be produced. In 1953 Salk and his co-workers prepared a poliomyelitis vaccine by growing three serotypes of the virus in monkey kidney cells followed by inactivation with formaldehyde. At the same time Sabin, Koprowski and Cox developed live attenuated poliovaccines for oral administration which became the vaccine of choice and has been used worldwide since 1959, mainly because it is the easier of the two to administer. It does however have the disadvantage of causing a small number of cases of paralysis in recipients or contacts as the vaccine strain spontaneously reverts to the wild type virus at a very low rate (135, 156).

Combining several vaccines into a single formulation for immunization against different diseases has been commonly used. Successful examples include that of polioviruses serotypes 1, 2 and 3 into a single vaccine against poliomyelitis and the combination of measles, mumps and rubella vaccines into a triple vaccine. In 1978 the island of Grenada claimed, by using the "Dermojet" gun and combined vaccines, to be the first nation in the world to have 100% of its children immunized against diphtheria, whooping cough, tetanus, polio, tuberculosis, measles and typhoid (50). The use of combined vaccines offers a number of obvious advantages, the first being a decrease in the number of vaccine inoculations and consequent clinic visits which should result in an increased compliance to vaccination schedules. Secondly, combining vaccines reduces the cost of storage, transport and administration. Thirdly, combining vaccines simplifies vaccine schedules, facilitating record keeping and providing the opportunity for a universal approach to global vaccination (153).

Attenuation can be defined as a method for reducing the virulence of a live virus vaccine. Vaccine efficacy refers to its effectiveness under optimized conditions. Traditionally, vaccines have been attenuated by either serial passage in tissue culture or inactivation by physical or chemical means. Although such vaccines have been and remain effective they do possess several disadvantages. The genetic modifications responsible for attenuation are often undefined leaving the possibility of reversion and possible vaccine induced disease (45). When producing killed vaccines, great care must be taken to ensure that each batch produced has been inactivated sufficiently so that virulence is reduced but protective efficacy is not lost. This presents considerable challenges to the manufacturer (35).

Advances in recombinant DNA technology, molecular biology and immunology potentially enables a vaccine against any infectious disease to be developed, given sufficient resources, and should eliminate many of the problems associated with traditionally produced vaccines (97).

The principle directions in which vaccine development is now being pursued include:

1. Purified component vaccines (35, 45)
2. Synthetic antigens (6, 35, 190)
3. Neoglycoconjugates (35)
4. Genetically modified strains (35, 113)
5. Nucleic acid vaccines (35, 170)
6. Novel antigen delivery systems (35)

The most recent developments with measles vaccine have concentrated on aerosol delivery. This form of delivery is painless, rapid and non-invasive and therefore suitable for mass campaigns. Results in comparison to vaccine given subcutaneously suggest the aerosol route evokes a better humoral response which remains significantly higher, and may also induce superior mucosal immunity (46). Since this technique also avoids the risks associated with injections, aerosol delivery of measles vaccine could therefore help with the eradication of measles.

The World Health Organization (WHO), the Expanded Program on Immunization (EPI) and the Global Program on Vaccination (GPV)

During the late 19th century it became clear that current vaccination strategies were not adequate to control smallpox as evident from its incidence which, though in decline, persisted. During the 1950s eradication efforts were intensified and smallpox was eliminated from several regions of the world but was still endemic in 31 countries, prompting the 1958 World Health Assembly to propose the idea of global eradication. The World Health Organization began work on this project in 1967 and, on 8th May 1980, the 33rd World Health Assembly was able to declare smallpox eradicated (191).

When it was apparent that the smallpox campaign was going to succeed, the WHO initiated the Expanded Program on Immunization (EPI) whose aim was to deliver on a global basis vaccines for diphtheria, pertussis, tetanus, poliomyelitis, measles and BCG for tuberculosis. Later it was decided to include hepatitis B and yellow fever for those countries where it occurs. National Immunization Days are encouraged where all children under 5 years of age are immunized regardless of previous vaccination history which is an effective way of catching children who miss routine infant immunization (130).

Following progress that has been made with EPI the WHO decided, in 1993, that if vaccination was to be used efficiently and effectively to combat infectious disease on a world wide scale the following needed to be carefully coordinated and managed as a whole: research and development; liaison with industry; the introduction of new vaccines into EPI; securing a stable supply of quality vaccines and monitoring the progress of the control of various diseases (90). Therefore the Global Program on Vaccination (GPV) was created which combined two existing programs, EPI and

Vaccine Research and Development, with one new program on Vaccine Supply and Quality Control. GPV has four goals (90):

1. to aid development of new vaccines which can be incorporated into EPI extending immunization to diseases not currently vaccine preventable but which cause significant morbidity and mortality in developing countries.
2. the development of new vaccination approaches so simplifying the immunization process using combined vaccines and alternative routes of administration and to improve vaccination efficiency using novel immunization approaches such as nucleic acid vaccines and new adjuvants.
3. to introduce new vaccines into EPI and evaluate their efficacy in field trials and advise on immunization strategies.
4. the development of simple diagnostic tools to monitor the progress and effectiveness in EPI.

The work described in this thesis has a direct relevance to the goals of GPV, particularly the fourth, since one aim of the study is to develop simple assays which use oral fluids as a substitute for serum to measure IgG responses to vaccine preventable virus infections. The use of oral fluids allows larger study populations and those populations previously inaccessible to be reached. Coupled with simple specific assays suitable for field use, this will enable the progress and effectiveness of viral vaccines within EPI to be monitored more easily and widely. Two vaccine preventable virus infections, rubella and hepatitis B, were investigated in studies described in this thesis.

RUBELLA

Historical Perspective

Postnatal rubella is a mild illness and for many years was considered to be a disease of minor importance. Rubella was first described as a disease distinct from other exanthemata by the German physician de Bergen in the early 1800s and was termed "Rotheln". The first person to describe rubella in the UK was Dr. William Maton who reported an outbreak of an illness resembling a mild form of scarlatina in a boys public school in 1815. Several years later Maton again observed an outbreak of a rash associated illness and was able to describe the characteristic clinical features of rubella including mild illness followed by a generalized, often tingling rash lasting for a few days with enlarged and tender glands. In 1866 Dr. Henry Veale, a Scottish physician, described in the Edinburgh Medical Journal 30 cases of rubella (184) and as an alternative to Rotheln, proposed the Latin name "rubella" as an easily pronounceable alternative, meaning "little red" (184). (Reviewed in (34), (58), (109), (195)).

For almost a century rubella received very little attention until, in 1941, an Australian ophthalmologist named Norman Gregg made a very important finding following a rubella outbreak in New South Wales. He reported an association between rubella in pregnancy and congenital abnormalities, many of which were serious (66). Initially his findings were met with skepticism but during the next twenty years his observations were confirmed by others (189)(reviewed in (195)). A pandemic of rubella began in Europe in the early 1960s and spread to the United States which experienced a huge rubella epidemic in 1964/5 resulting in an estimated 20,000 rubella damaged babies. This led to a better understanding of congenital rubella

syndrome and emphasized the need to develop a vaccine and suitable immunization programme (33, 132) (reviewed in (71)).

Biology and virological properties

Rubella virus (RV) is the sole member of the genus *rubivirus* which is classified within the *Togaviridae* family. Unlike other members of the Togaviridae family, RV has no known invertebrate host with man being the only known natural reservoir. Electron microscopy shows RV virions to be small, pleomorphic, 50-70 nm in diameter, and consist of a 30 nm electron dense core surrounded by an envelope (134). The genome is a single strand of positive sense RNA. It is encapsulated in an icosahedral protein shell (T = 4) (24) composed of the capsid protein (C), enveloped by a lipid bilayer derived from the host cell plasma membrane (8). Projecting through the envelope are viral encoded glycoproteins E1 and E2. These are thought to interact with host cell receptors during adsorption and much of the hosts immune response is directed against them (167).

The positive sense single stranded RNA genome is 9,762 nucleotides in length (93) and is arranged into two parts: the 5` end codes for nonstructural proteins needed for the transcription and translation of RNA; the 3` end codes for the capsid (C), E1 and E2 structural proteins (see page 21) (reviewed in (56, 93)). During replication a discrete subgenomic mRNA species, identical to the 3` terminal sequence of the genomic RNA is synthesized, capped and polyadenylated and acts as the mRNA for the synthesis of viral structural proteins (reviewed in (167)). A number of cotranslational and post translational modifications occur to the rubella glycoproteins before they are incorporated into virus particles (reviewed in (167, 195)).

Clinical Features of Rubella

Rubella virus is spread by the respiratory route. Infection may be asymptomatic or cause clinical symptoms which include a maculopapular rash, lymphadenopathy, low grade fever, conjunctivitis, sore throat and arthralgia. The rash is the most prominent feature, usually appearing on the face and spreading outwards. Symptoms are preceded by an incubation period of between 7 and 9 days followed by the appearance of virus in the serum after which virus is shed into the nasopharynx. The maculopapular rash begins 13-21 days after exposure and coincides with the detection of rubella specific antibody suggesting the rash may be an immune mediated phenomenon. An IgM antibody response predominates at the onset of illness and within a week antibodies to all immunoglobulin classes are detectable (reviewed in (195)).

Congenital Rubella Syndrome

In contrast to postnatal rubella, rubella virus infection acquired during pregnancy often has severe consequences because fetal development may be impaired giving rise to the congenital rubella syndrome (CRS) (reviewed in (195)). The early fetus (embryo) is at risk from rubella infection very shortly after conception. Some infected fetuses can escape damage but fetal death is a considerable risk following infection before the 16th week of gestation and 67-85% of neonates show clinical sequelae following maternal infection in the first trimester (121). Maternal infection after the second trimester does not usually result in fetal damage. The effects of fetal rubella range from those that are obvious at birth such as neonatal cataracts and other physical defects to those which do not become clinically apparent for several years including

retinopathy, mental retardation and hearing loss. In developing countries, fetuses with severe multiple defects probably die unrecognized during pregnancy or as infants soon after birth (reviewed in (36, 108, 118, 195)).

The mechanisms of rubella virus teratogenesis remain unknown and suggestions include the possibility of damage due to viral replication in focal clones of cells at critical stages of the development of fetal organs. The prolonged persistence of rubella virus in fetal tissues suggests that neither maternal IgG nor the developing fetal immune system can eliminate the virus *in utero* despite the ability of both maternal IgG and fetal IgM to neutralize the virus *in vitro* (106). How the virus is able to escape and establish persistent infections in the fetus is yet to be determined (195) and may possibly be related to the ability of RV to mature in intracellular vacuoles and so avoid the immune system.

In 1984, the WHO Regional Committee for Europe set the year 2000 as a target date for the elimination of congenital rubella from the Region. It was decided that by 1990 all European countries should include rubella vaccine in their national programs, by 1995 should have achieved at least 90% coverage of the target population and by 1996 should have investigated every case of congenital rubella syndrome (58).

Epidemiology

Since man is the only known host for rubella virus, the virus must continually cycle within the human population for perpetuation of the disease. As infection with rubella virus is often sub clinical or symptoms being generally mild, clinical diagnosis may be unreliable so compromising the accuracy of studies of rubella incidence. Medical

attention is often not sought and even when it is, laboratory confirmation is often not obtained nor a specific diagnosis made. Seroepidemiological studies have been used to provide more representative data and, despite the difficulty of obtaining completely reliable information, it seems likely that rubella is endemic worldwide. Before the advent of rubella vaccination, pools of susceptibles accumulated in such a way as to give rise to epidemics at intervals of 3-9 years in different geographic locations with major world epidemics every 10-30 years. In some European countries epidemics were recorded at more frequent intervals than in the USA. In Taiwan, rubella was not endemic but occurred in epidemic cycles at ten year intervals (59). A major problem in identifying epidemics of rubella, particularly in developing countries with poor health-care facilities, is the lack of apparent clinical symptoms which may cause rubella outbreaks to pass unrecognized (195).

The age at which rubella virus infection occurs varies geographically. For the majority of industrialized nations with temperate climates infection peaks among young school children between the ages of five and nine years. In contrast, a considerably higher proportion of this age group are seropositive in equatorial countries, suggesting a higher attack rate amongst preschool children. In much of Africa the majority of children are immune by the age of 10 years. Population densities and socioeconomic factors greatly influence the appearance and spread of epidemics and serology has been used to show that rubella infection is highest amongst the densely housed (43, 109, 161).

Populations considered to be at particular risk, especially amongst child-bearing age-groups, are island communities and migrant populations from rural areas. This is due

to the lower proportion of seropositive individuals within these groups as a result of a lower risk of exposure to rubella during childhood (108, 110).

Rubella Vaccines

Attempts to produce a chemically inactivated rubella vaccine during the early 1960s were unsuccessful and were discontinued when it was reported that rubella virus could be attenuated by multiple passage in tissue culture. The first attenuated strain of rubella virus was produced using an isolate from a US military recruit in 1961 (73). It was passaged 77 times in vervet monkey kidney cell cultures to give a prototype vaccine referred to as high-passage virus-77 or HPV77. This was then given a further 5 passages in duck embryo fibroblasts as they are thought less likely to contain extraneous agents. This strain was called HPV77. DE5 (73) and was introduced for use in many European countries and the United States in 1969/70. Another vaccine strain, DK12, was produced by passaging HPV77 12 times in dog kidney cell cultures and was also licensed in 1969 but its use was discontinued as it caused an unacceptably high number of adverse reactions after administration (173).

Another attenuated rubella vaccine, the Cendehill strain, was licensed for use in the USA and UK in 1969 and 1970 respectively. This strain was isolated from a urine sample from a case of postnatally aquired rubella and attenuated by 51 passages in primary rabbit kidney cultures (77). A further attenuated rubella vaccine, the RA27/3 strain was licensed for use in the UK in 1972 and is now the most widely used rubella vaccine strain. The name RA27/3 refers to "rubella abortus, 27th specimen, third explant" and the vaccine strain was derived from the kidney of a rubella infected fetus and attenuated by 4 passages in human embryonic kidney cells and 17-25

passages in human diploid cell culture (WI-38 fibroblasts), avoiding the use of nonhuman cells (147). RA27/3 replaced the HPV77. DE5 vaccine strain in the USA in 1979 and is currently incorporated into the MMR vaccines produced in the USA and Western Europe as the antibody response and protection it affords most closely resembles that induced by naturally aquired infections. Another five attenuated strains of rubella virus have been developed, four of which are used in Japan and the fifth, the BRD-2 vaccine strain, has been developed in China (reviewed in (11) and (71)). A summary of rubella vaccine strains is shown in table 1.

Table 1. Origin and passage history of attenuated rubella virus vaccine strains (11)

Strain designation	Origin of strain	Attenuation/Passage history in cell cultures
HPV77	US Army recruit	VMK (77)
HPV77.DE5	As above	VMK (77); duck embryo (5)
Cendehill	Urine from case of post natal rubella	VMK (3); primary rabbit kidney
RA27/3	Kidney of rubella infected fetus	Human embryonic kidney (4); WI-38 fibroblasts (17-25)
DCRB 19	Throat swab from patient in Tokyo	VMK (1); Bovine kidney (53); rabbit kidney (3)
KRT	Throat swab from patient in Matsue, Japan	VMK (4); primary rabbit testicle (36); primary rabbit kidney (1)
MEQ_{11}	Throat washing from patient in Osaka, Japan	VMK (14); Chick amnion (65); quail embryo fibroblasts (11)
TO-336	Pharyngeal secretion from post natally infected child, Toyama, Japan	VMK (7); primary guinea pig kidney (20); primary rabbit kidney (3)
SK	Throat washing from patient in Kumamato, Japan	VMK (1); swine kidney (60); rabbit kidney (6)
BRD-2	Child with post natal rubella, China	Human diploid cells (30)

VMK = vervet monkey kidney, (x) = number of passages

Rubella Immunisation Programmes

Rubella immunization programs are designed to prevent maternal rubella infections and the subsequent teratogenic effects on the fetus. The development of rubella vaccines has created a unique situation in that the primary group of people who contract and spread post natal rubella differ from those who benefit most from the vaccine, namely the unborn children of pregnant mothers. Consequently ethical consideration has led to different countries using different immunization strategies so the achievement of the main goal of vaccination has varied (102, 103).

Despite the availability of rubella vaccine, it has not been recommended for use in EPI in developing countries. This is mainly because a sustained high coverage cannot be guaranteed in such countries so a strong possibility exists for the slowing rather than complete interruption of rubella transmission. This would result in an increase in the average age of infection therefore increasing the suceptability of adult women of child bearing age. However, many industrialized countries include rubella vaccine in their programmes and approximately a third of all countries worldwide report a national policy of rubella vaccination (141).

Three strategies for rubella vaccination have been used (58, 138, 161):

1. The first approach was used in the USA and started with a mass vaccination of children under 12 years of age and was followed by routine vaccination of children of both sexes at the age of one year. This approach is designed to interrupt transmission of rubella virus amongst young children, who represent the primary transmission group, so minimising the possibility of exposure of susceptible pregnant women. This strategy requires a very high vaccine coverage in excess of

90%. Rubella vaccine is delivered as part of the combined MMR vaccine and sufficient coverage is maintained in the USA as a result of the USA school immunization law which requires documentary evidence of immunization against specific diseases prior to school entry.

2. A second approach was used in the UK until 1988 and has also been used in some other European countries. It is a selective approach targeting prepubertal girls and non-immune women, either before or after pregnancy, with one dose of monovalent rubella vaccine. The aim of this strategy is to protect individuals and prevent maternal rubella infections rather than to diminish natural circulation of the wild type virus which is thought to contribute to maintaining the level of immunity in women of child-bearing age. This approach is particularly appropriate where there is evidence to suggest that vaccine coverage in mass campaigns is poor. Since a rubella epidemic in 1986, the vaccination programme in the UK was expanded in 1988 to include preschool children of both sexes with rubella being included in a combined MMR vaccine.
3. The third approach was first successfully implemented in Sweden. It was introduced in 1982 and involves a two-dose schedule using a combined MMR vaccine. Vaccination is optional, free of charge and is offered to children at 18 months and again at 12 years of age with the second dose given irrespective of history of disease or previous vaccination. By immunizing those not vaccinated at 18 months and those who responded poorly to the first dose of vaccine this programme is designed to prevent an accumulation of susceptibles in older age groups, and the ultimate aim of this strategy is to eliminate rubella as well as measles and mumps.

Epidemiological evidence suggests that whilst selective vaccination strategies can achieve a considerable reduction in the incidence of rubella infection in pregnancy the risk still remains unacceptably high as the strategy is designed to enable some circulation of natural rubella virus amongst non-immunised children and adults. Therefore selective immunization strategies are being replaced by combined strategies which include mass immunization of young children which quickly reduces the circulation of rubella virus, and the effectiveness of this approach is demonstrated by the considerable reduction in CRS cases in the UK between 1971 and 1996 (182). These strategies are, however, not without problems and mathematical models predict that vaccine coverage must exceed at least 70% to avoid large pools of susceptibles in older age groups and therefore a potential risk of CRS. Several approaches are used to maintain vaccination coverage at a suitably high level including an increased professional commitment towards vaccination, adequate amounts of vaccine and funding and the introduction of legislation that requires evidence of immunization for entry to school as in the USA.

If a mother is inadvertently immunized with rubella vaccine during early pregnancy a concern is any risk this poses to the developing fetus. Of 712 documented cases of susceptible women vaccinated during pregnancy, none of the resulting children have shown malformations compatible with congenital rubella syndrome. This suggests the vaccine strains of rubella virus do not have the teratogenic capacity of natural rubella virus (9, 148).

Due to the potential problems that are associated with insufficient vaccine coverage it is important that rubella control programmes have built in monitoring systems.

Vaccine coverage of target age-groups should be monitored and in countries using MMR vaccine, measles, mumps and rubella should all be notifiable diseases (116, 164, 194).

Laboratory diagnosis

Due to the difficulties associated with accurate clinical diagnosis and because viral cell culture is difficult and time consuming, serological investigation remains the diagnostic method of choice. Accurate laboratory diagnosis of past or recent rubella is essential both for the individual patient, especially the woman presenting with a rash (or history of contact with rash) in early pregnancy, and for the design and monitoring of vaccination programmes. Screening for rubella antibody is also important to ascertain whether immunization should be advised, though only pregnant women are universally screened. The current policy is to recommend rubella immunization should level of rubella specific IgG antibody be lower than 10 IU/mL, which is considered to be the minimum protective level of antibody (172). Previously, 15 IU/ml was considered to be the minimum protective level (117). However, in 1992 the Rubella Subcommittee of the National Committee for Clinical Laboratory Standards in the USA elected to use 10 IU/ml as the breakpoint to define rubella immunity. This recommendation was based upon epidemiological studies, anecdotal reports and the already widespread use of this lower limit in the United States without apparent adverse effect (172).

Classical serological tests such as the complement fixation test (CFT), indirect immunofluoresence (IF) and haemagglutination-inhibition (HI) were introduced for the laboratory diagnosis of rubella in the late 1960s, with the HI test becoming the

assay of choice as it used a commercially available freeze dried antigen and could reliably detect all classes of rubella specific antibody. The HI test is, however, technically demanding and time consuming so is no longer recommended for rubella antibody screening but despite its problems, remains the reference standard against which newer assays are compared. Subsequently, in the UK, radial haemolysis (RH) as described by Kurtz et al (89)was introduced to meet the need for a simple and economical screening test which was both sensitive and specific in anticipation of a national rubella vaccination campaign. Red cell-based assays, however, are now being superseded by commercially available enzyme linked immunosorbent assays (ELISA), which can detect rubella specific IgG and IgM antibody and are highly sensitive and specific. Latex agglutination (LA) is also recommended as a rapid and sensitive screening assay for immunity. In reference laboratories, radioimmunoassays (RIA) have been used, especially those with IgM capture formats which detect the proportion of IgM specific for rubella (37, 104, 195).

THE MOLECULAR BIOLOGY OF RUBELLA VIRUS

The genomic structure of rubella

The RV genome is 9,762 nucleotides in length, excluding a poly adenosine (A) tract situated terminally at the 3' end. The base composition is 14.9% adenosine (A), 15.4% uracil (U), 30.8% guanosine (G) and 38.7% cytidine (C), with the total rubella G + C content of 69.5% being the highest of any RNA virus sequenced to date. Semliki Forest virus, also a member of the *Togaviridae* family, with a G + C content of 53% is the RNA virus with the next highest G + C content so far determined (reviewed in (56, 167)). The unusually high G + C content of the RV genome presents

a difficulty for both the design of specific primer sequences for polymerase chain reaction (PCR) assays and sequence determination of the RV genome (17, 25, 48, 57).

RNA is labile and vulnerable to the effects of degredative enzymes such as ribonuclease (RNase). The RV genome is no exception. This suggests that virion RNA may be exposed on or near the surface of the capsid or that RNase can easily penetrate into the capsid. Alternatively, virion RNA may form a structural component of the capsid so making it vulnerable to the effects of RNase (56). The fact that the RV genome is labile and sensitive to the effects of RNase has implications for the collection of samples and this is addressed later. When collecting samples for RNA testing important considerations are therefore any prior treatments to which a sample may have been subjected, the manner in which samples have been stored and the number of freeze thawing cycles a sample may have undergone.

The RV genome contains two non-overlapping open reading frames (ORFs), as shown in Fig. 1.

The genomic organisation of rubella virus (195)

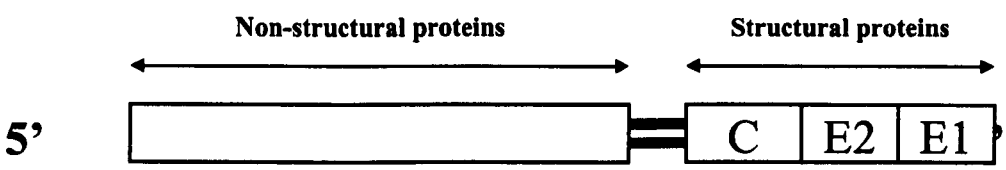


Fig. 1

The 5' ORF encompasses approximately two thirds of the genome being 6345 nucleotides in length and encodes non-structural proteins involved in viral RNA replication. The second ORF is 3' proximal and is 3189 nucleotides long encompassing the other one third of the genome, encoding three virion proteins consisting of the nucleocapsid (C) and the glycoproteins E1 and E2. Both these glycoproteins are described as class I membrane proteins in that they contain a single potential transmembrane sequence. Both are further classified as type I glycoproteins as the predicted transmembrane sequence is near the carboxy terminus, in contrast to type II glycoproteins where the transmembrane sequence is found near the amino terminus. E1 and E2 are 481 and 282 amino acids in length respectively, and of the two, E2 is more heavily glycosylated. The function of these glycoproteins is thought to be involved with the binding of RV to specific host cell surface receptors (reviewed in (12, 56)).

Electron microscopy suggests that E1 and E2 project from the RV lipid envelope to form surface spikes approximately 6 - 8 nm in length. Further studies using antisera specific for E1 and E2 after non-ionic detergents have been used to disrupt the RV virion suggest that E1 and E2 form a heterodimer complex stabalized by multiple inter chain disulphide bonds (reviewed in (56)). Studies show that the majority of E2 is poorly accessible at the surface of the virion to the actions of glycosidases, neuraminidase and trypsin and is inaccessible to monoclonal antibodies specifically reactive with the amino-terminus region (reviewed in (195)). Therefore it is likely that E1 is the more exposed of the two glycoproteins in the herterodimer. A humoral response in the host is induced by both E1 and E2 with E1 being immunodominant. A

number of linear epitopes recognised by murine monoclonal antibodies, some of which are neutralising, have been mapped between amino acids 210 and 286 of the total of 481 that make up E1 and RV immune human sera have been shown to react with this region (56).

Genomic sequencing

The basis for sequence variation in the nucleotides of both RNA and DNA viruses is thought to be that genomic variation enables viruses to adapt to changes in the environment and conditions of replication. Such nucleotide variation may lead to changes in the amino acid composition of the virion which is often reflected as antigenic variation. Alternatively, as is often the case, there is no structural change, the sequence variation helping the virus to optimise its replication. The advent and development of the polymerase chain reaction (PCR), automated sequencing technology and the necessary computer software needed to analyse the resulting data has opened up the possibility of studying the molecular epidemiology of viruses (107). Such techniques have recently been successfully used to investigate the molecular epidemiology of measles virus, an important vaccine preventable virus infection (80, 81).

The investigation of genetic heterogeneity by genomic sequencing has important implications for both epidemiologists and clinicians. Firstly, molecular studies of important antigenic regions may lead to the characterisation of a new strain antigenically different from strains currently known to be circulating and so disclose the presence of a virus with the potential for transmission within a population considered immune. This is particularly important for vaccine preventable virus

infections. Secondly, a detailed knowledge of virus nucleotide sequence and its genetic heterogeneity enables the tracking of chains of transmission of infection which can lead to the tracing of the origin of infection in an outbreak and investigation of unusual cases (143).

Rubella PCR

When developing a suitable PCR-based assay for the detection of RV RNA there are several technical aspects which need consideration. Firstly, the RV genome consists of RNA and so must be reverse transcribed to copy DNA (cDNA). Secondly, to obtain a necessary level of sensitivity a double amplification or nested PCR is needed where the initial amplified sequence, or first round product, is re-amplified to produce a second round product in sufficient quantities for visualisation by staining with ethidium bromide after electrophoresis on an agarose gel .

The choice of primers is critical when designing a PCR assay. This is largely dependant upon the specific purpose for which the PCR assay is used such as clinical diagnosis or investigating molecular epidemiology. Any information on the nucleotide sequence of the virus in question that is already known should be taken into account. Each primer should ideally be 20-30 nucleotides in length, chosen within a nucleotide region of the virus genome that is known to be well conserved and contain an optimal ratio of G+C and A+T residues. Ideally the G+C content of a primer should be within the range of 35-55% of the total nucleotides making up the primer (30, 163). The length of nested PCR product that is generated between the second round 3' and 5' primers should be chosen to suit the purposes of the PCR assay. If the PCR is required for diagnosis only it is not necessary to generate a large product, the determining

factors being those needed to produce a result with maximum sensitivity and specificity, speed, and efficiency. If, however, the PCR is to be used for molecular phylogeny it should be designed so that a substantially longer second round product is produced to enable differences in nucleotide sequence of the genome to be observed and their significance determined. However, choosing a product greater than 700 nucleotides in length may result in decreased efficiency of the PCR assay. To determine the extent of genomic heterogeneity it is essential that the region of genome chosen for PCR is one that encodes amino acids on which there is sufficient selection pressure to produce variation, in particular antigenic variation (56, 57, 79, 81, 86, 107).

Of the three structural proteins of RV, the E1 glycoprotein is the most exposed antigen and the majority of the host's humoral response appear to be directed against it. Since the information on RV genome variation is limited, a reasonable assumption has been made that there is most pressure on the E1 glycoprotein to vary. Of the genes making up the two RV ORFs it is probable, therefore, that most nucleotide variation will be seen within the E1 gene. Therefore, when developing a PCR based assay specifically for investigating the molecular epidemiology of rubella, primers should be designed that encompass a region of the E1 gene. They should ideally include the region between amino acids 210 and 286 which encodes epitopes against which RV immune human serum has been shown to react (56, 57, 143, 195, 196).

Three published PCR assays have been described for detecting RV RNA for the clinical diagnosis of fetal rubella infection. All three target a region of the E1 gene and produce a relatively short second round product adequate for diagnosis but of relatively little value for molecular epidemiological studies. That described by Bosma

et al (18) produces a second round product 143 nucleotides in length. Revello et al (155) described a PCR-assay with a second round product slightly smaller at 125 nucleotides in length whilst that described by Tanemura et al (175) produces a larger product of 283 nucleotides.

The molecular epidemiology of rubella

To date three studies have been published on the molecular epidemiology of rubella. The first was carried out by Bosma et al (17) in 1996 using a 309 nucleotide amplicon that targeted the E1 glycoprotein. A total of 22 rubella virus isolates from Europe, Asia and the USA obtained between 1963-1995 were used. Nucleotide sequence divergence for these strains ranged from 0.65% to 7.14%, with greatest divergence seen in two rubella viruses of Indian origin compared to the Therien strain. The second study was conducted by Katow et al (86) and used 26 strains of rubella virus. The majority were obtained from Japan, China and Hong Kong including the vaccine strains TCRB19 and To-336 from Japan and the BRD2 vaccine strain produced in 1980 by the National Vaccine and Serum Institute in Beijing, China. Three strains were obtained from the USA and two from the UK. The PCR amplification and sequencing protocol used was developed to determine the sequence of 1483 nucleotides of the E1 gene. This study found that the interstrain variation of the nucleotide sequence of the RV genome (0.1% - 10%) was comparable to that found for other RNA viruses (e.g. 0.5% - 7.2% in measles virus and 0.1%-6.6% in influenza type C). The study yielded several other points of interest. Firstly, of three Chinese strains, two - the BRD2 vaccine strain and a strain isolated from an adult from Hong Kong in the 1980s - showed considerable diversity from the other 24 strains. Secondly, all strains analysed which were isolated in the 1960s - which included five

isolates from Japan and all those from the USA and UK - were classified into a single group which suggests that this genotype may have been involved in a worldwide epidemic. A further strain isolated from a case of CRS in Hong Kong in 1985 was closely related to strains isolated from north eastern Japan. Lastly, two strains isolated from Japan during 1990 were assigned to different positions on a phylogenetic tree suggesting that at least two RV strains were co-circulating in Japan during this period.

The third study was conducted by Frey et al (57) and is the largest of the three. It describes the molecular epidemiology of RV using 63 isolates from North America, Europe and Asia collected between 1961 and 1997. The study is based on sequence analysis of the entire E1 gene, determined using PCR amplification of six fragments encompassing the whole gene using pairs of internal overlapping primers. The collection of rubella viruses sequenced included all the strains whose complete E1 sequences had been described in previous studies (17, 86). The RV strains were divided into two genotypes which differed from each other by 8% - 10% in their nucleotide sequences. Those designated genotype I consisted of the majority of the virus isolates in the collection and contained strains from Europe, North America and Japan. The second genotype, genotype II, was represented by three isolates - two from China and one from India - with the diversity of the two Chinese isolates having been previously noted by Katow et al (86). E1 amino acid sequences were observed to differ by no more than 3% which suggests that RV is not prone to major antigenic variation within this gene.

The geographical range of isolates used to date to study the molecular epidemiology of rubella is limited and both genotypes, particularly genotype II, need to be

investigated further using samples from all continents of the world. Isolates from South America, Africa and Australia have yet to be sequenced and a minimal number of isolates from China and India are currently represented. Further studies should determine the geographic extent of the two genotypes and whether other genotypes exist.

HEPATITIS B

Historical background

Australia antigen, now referred to as hepatitis B surface antigen (HBsAg), was discovered by Dr. Baruch Blumberg in 1965 (13, 15). He observed an immunodiffusion precipitin line between HBsAg in the serum of an Australian Aborigine and HBsAg antibody present in the serum of a hemophiliac who had received multiple transfusions. The link to clinical illness was made when a laboratory technician working with Australia antigen developed acute hepatitis, and rapid progress in the understanding of hepatitis B virus (HBV) has subsequently been made, reviewed by Lee, 1997 (94). Infection with HBV results in a broad range of liver disease ranging from sub-clinical infection to acute or chronic hepatitis, cirrhosis, and hepatocellular carcinoma and accounts for approximately one million deaths annually worldwide (94, 115). Early evidence suggesting that the liver damage sustained as a result of HBV infection is immune mediated as a consequence of the cellular immune response against viral antigens present in infected hepatocytes has been confirmed (95) (reviewed in (92, 115)). The WHO estimate that the world population is currently approximately 5000 million and of these, about 2000 million people have serological markers of HBV infection (84).

The carrier state

The carrier state is defined as the persistence of HBsAg in the circulation for at least six months. It may be lifelong and is associated with liver damage ranging from minor changes in the nuclei of hepatocytes to hepatocellular carcinoma. The carrier state ensures the survival of HBV and is established in approximately 5-10 % of infected adults (82, 175). The WHO estimate there to be 350 million chronic carriers

worldwide, of whom approximately 65 million will die as a result of liver disease (85) (reviewed in (84)).

Epidemiology

The distribution of HBV infection varies greatly worldwide. The prevalence is very high in Southeast Asia, China and sub-Saharan Africa where over half the population will be infected at some time in their lives and over 8 percent of the general population are chronic carriers of the virus. In these areas the majority of infection occurs during childhood, either from an infected mother to her baby (vertical transmission) or from one child to another (horizontal transmission). In contrast, North America, Western Europe and Australia (amongst persons of European decent) are areas of low endemicity where only a minority of people come into contact with the virus mainly as a result of horizontal transmission between young adults from sexual activity, injection-drug use, or occupational exposure (reviewed in (84, 94)). HBV may be present in serum in large quantities, with up to 10^9 virions per milliliter. It can also be detected in saliva and semen and there is much evidence to suggest transmission of HBV occurs by intimate contact and the sexual route (reviewed in (94)). Therefore, on a global basis, HBV can be regarded as one of the most important infectious diseases in the world and should be viewed as no less important than any other vaccine-preventable diseases in view of the number of people affected and severity of liver disease (reviewed in (84)).

Biology and virological properties

HBV belongs to a family of closely related DNA viruses called the *hepadnaviridae* which includes the woodchuck hepatitis virus, the duck hepatitis B virus and several

other avian and mammalian viruses. All *hepadnaviridae* have a similar hepatotropism and life cycle within their hosts and can be a cause of hepatocellular carcinoma (61, 76).

The genome of HBV is a partially double stranded circular DNA molecule. With a size of approximately 3200 base pairs it is one of the smallest DNA viruses known. One strand, almost a complete circle, is termed "minus", and the other "plus", which is shorter and variable in length. The HBV genome has four overlapping open reading frames encoding the surface (S) and core (C) structural genes and the replicative polymerase (P) and X gene, which encodes two proteins that act as transcriptional transactivators. The S and C genes have upstream regions termed *pre*S and *pre*C (reviewed in (92, 94)).

Electron microscopy of HBV positive serum shows three morphologically distinct forms of particle. Two forms of particle are characteristically present in excess as small spheres and tubular structures, both approximately 20 nm in diameter. The third type of particle, the whole virion, is seen as a spherical double shelled structure, 42 nm in diameter and is referred to as the Dane particle after its discoverer (44, 76). All three particles have a common antigen on their surface, termed hepatitis B surface antigen (HBsAg), which is present at a high concentration in the serum of infected hosts (reviewed in (61, 76, 94)).

The Dane particle, which is the infectious virion of HBV, is comprised of the genome enclosed by a nucleocapsid, the major structural protein of which is hepatitis B core antigen (HBcAg). In turn, the nucleocapsid is enclosed by a lipoprotein envelope

containing the viral surface proteins, including HBsAg. A third protein, the "e" antigen (HBeAg), is found in soluble form in virus-positive sera and is a peptide derived from the core antigen (reviewed in (76, 94)).

Diagnosis and immunology of HBV

The presence of HBV DNA and antigens or their antibodies can be used as diagnostic markers to determine the status of HBV infection, both current and past (Table 2). One indicator of active viral replication is the presence of HBV DNA in serum which can be detected by hybridisation methods or by sensitive polymerase-chain-reaction (PCR) techniques (76, 94). However, HBsAg is more often used as a marker of current infection since its presence in the blood, sometimes in large amounts, is often the first sign of HBV infection and current technology for its detection is cheaper and simpler than that for HBV DNA. In acute infections HBsAg levels decline and it is eventually replaced by antibody to HBsAg (anti-HBs) which is assumed to be produced during the earliest stages of infection but remains undetectable because of binding to excess HBsAg. Progession to the chronic state is associated with the persistence of HBsAg with little change in concentration in the serum (75). Anti-HBs confers protective immunity and is detectable in patients who have recovered from HBV infection and in persons immunized with HBV vaccine. However, anti-HBs is not always long lasting and may become undetectable in patients who have made a full recovery from infection (76).

In contrast to HBsAg, free HBcAg is rarely seen in HBV infected patients since the nucleocapsid is surrounded by HBsAg. When HBcAg derived peptides are expressed on the surface of hepatocytes an immune response is induced which kills infected

cells. Antibody to HBcAg (anti-HBc) does not confer protective immunity though anti-HBc IgG is long lasting and can be detected in almost all patients who have been exposed to HBV so can be used as a marker of previous HBV infection. Anti-HBc is also present in patients with acute and chronic HBV infection and in acute disease is predominantly of the IgM immunoglobulin class which remains detectable for six to eight months and is gradually replaced by IgG. However, the detection of IgM cannot be used as an entirely reliable marker of acute illness since patients with chronic HBV may become anti-HBc IgM positive during flares in their disease (76).

The hepatitis B e antigen (HBeAg) serves as a marker of active viral replication and is present only in persons with circulating serum HBV DNA. In acute hepatitis B, antibody to HBeAg (anti-HBe) replaces HBeAg during the early stages of convalesence which signals a reduction in viral replication and a beginning of the resolution of disease (75, 76, 94).

Table 2. Interpretation of diagnostic markers in patients with hepatitis B virus infection, modified from Zuckerman et al 1995 (198)

HBsAg	HBeAg	Anti-HBe	Anti-HBc		Anti-HBs	Interpretation
			IgM	IgG		
+	+	-	-	-	-	Incubation period
+	+	-	+	+	-	Acute HBV or carrier
+	+	-	-	+	-	Persistent carrier
+	-	+	+/-	+	-	Persistent carrier
-	-	+	+/-	+	+	Convalesence
-	-	-	-	+	+	Recovery
-	-	-	+	-	-	HBV infection, no detectable HBsAg
-	-	-	-	+	-	Recovery, loss of anti-HBs
-	-	-	-	-	+	Immunization; recovery with loss of anti-HBc; repeated exposure but no infection

Immunization against HBV

The current goals of HBV immunization are three fold: to prevent clinical disease, to prevent the development of chronic HBV and to prevent transmission of the virus (75). These are being met through the expanded programme for immunisation (EPI) and should result in a reduction in the number of cases of hepatocellular carcinoma.

Hepatitis B vaccines were introduced in the early 1980s and elicit the production of anti-HBs which confers protective immunity (96). Two types of vaccine have been widely used. The first is a plasma-derived vaccine (Heptavax B, Merck, Sharpe and Dohme, 1982) and consists of inactivated HBsAg particles purified from plasma of chronic carriers of HbsAg (47, 53, 60, 96). This vaccine was not widely accepted by the medical profession because it is derived directly from human plasma so may contain other human pathogens and is no longer produced in the USA. These issues prompted the development and production of a second type of vaccine developed by recombinant DNA technology (4, 47, 53). Until recently two recombinant vaccines were marketed. The first, called Recombivax HB, was produced by Merck, Sharpe and Dohme in 1986 and the second, called Engerix-B, was made by Smithkline Beecham Pharmaceuticals in 1989. Both are derived from yeast expression vectors transformed by plasmids bearing the HBsAg gene. They comprise non-glycosylated HBsAg particles that have been purified, adsorbed onto aluminium hydroxide, and preserved with thimerosal (53, 60, 96) (Recombivax HB. West Point, Pa.: Merck, 1995 [package insert]; Engerix-B. Philadelphia: SmithKline Beecham Pharmaceuticals, 1995 [package insert]). They represent the only products of

recombinant DNA technology so far commercially developed as vaccines against human viral disease (113).

The efficacy of both the plasma-derived and recombinant vaccines has been demonstrated by clinical trials using a three dose intramuscular immunization schedule which results in levels of antibody regarded as protective, defined as anti-HBs serum levels equivalent or greater than 10 mIU/mL (60). Since the introduction of HBV vaccines several immunization strategies have been adopted by different countries with varying degrees of success. While vaccination has been highly effective in decreasing the prevalence of HBV carriers, initial recommendations based on targeting high risk groups only failed to decrease the burden of hepatitis B disease. The current policy is one of universal immunization of infants in countries where there is a recognised risk of infection with HBV (14, 60). In the UK the prevalence of HBsAg is estimated to be less than 1% (72). However, the WHO has recommended that all countries adopt HBV vaccination into their respective national immunisation programmes (84). Therefore in the UK immunisation is recommended for people who are at risk of HBV infection which include the following groups (164):

- Babies born to mothers who are chronic carriers of HBV or to mothers who have had acute HBV during pregnancy
- Parenteral drug misusers
- Individuals who change sexual partners frequently
- Close family contacts of a case or carrier
- Families adopting children from countries with a high prevalence of HBV
- Haemophiliacs
- Patients with chronic renal failure

- Health care workers including students and trainees
- Staff and residents of residential accommodation for those with severe learning disabilities (mental handicap)
- Other occupational risk groups (e.g. morticians, enbalmers)
- Inmates of custodial institutions
- Those travelling to areas of high prevalence

From April 2000, health authorities in the UK are to ensure that all pregnant women are offered screening for HBV. This will enable the immunisation of babies born to infected mothers and reduce the risk of perinatal transmission (7, 52).

HBV vaccine is expensive and this has been a major problem in acheiving widespread use and a universal vaccination policy. This has made prevaccination screening an important issue which relates directly to the cost effective use of the vaccine (60).

Serological assays for HBV

Sensitive and specific laboratory tests such as enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) are now available for the detection of specific serological markers of infection with HBV and to assess the immune status of the patient. Many assays are licensed and the most commonly used now are ELISA rather than RIA in format.

Assays for HBsAg and anti-HBs : Assays are available which enable HBsAg to be detected in the 0.25 to 0.5 ng/mL range and anti-HBs down to a level of 1 mIU/mL.

The types of assay available for detecting HBsAg include RIA, ELISA and reverse passive hemagglutination (RPHA). Both RIA and ELISA work on a "sandwich" principle with an anti-HBs coated solid phase used to capture HBsAg which is then detected by an anti-HBs probe labelled with either ^{125}I (RIA) or horse-radish peroxidase (ELISA). A range of commercially available ELISAs are widely used to detect HBsAg. RPHA uses red blood cells coated with highly purified anti-HBs so the presence of HBsAg in the test serum will cause the red blood cells to agglutinate

For detecting anti-HBs, RIAs and ELISAs are also commonly used and are commercially available, again using the "sandwich" principle but with a HBsAg coated solid phase (76).

Assays for anti-HBc : assays detecting anti-HBc in both ELISA and RIA format are available and usually based upon a competitive or inhibition principle. Bound to the solid phase is HBcAg with which a labelled antibody and anti-HBc, if present in the serum sample, compete to bind. Hence the higher the concentration of anti-HBc in the sample, the lower the signal measured. Three types of assay measuring anti-HBc have been described: those that detect total anti-HBc antibody and those which detect anti-HBc IgG or IgM. A commercially available competitive radioimmunoassay (CORAB; Abbott Laboratories, North Chicago, Ill.) is sensitive and specific for the detection of total anti-HBc and the Murex ICE ELISA is an example of a commercial assay which detects total anti-HBc using a "sandwich" principle. Cohen et al (28) described a competitive RIA for detecting anti-HBc IgG and a commercially available ELISA, the Hepanostika anti-HBc Uni-Form kit produced by ORGANON Teknika, detects anti-HBc antibody using a similar competitive principle. Anti-HBc IgM can be detected

using a "sandwich" principle as described by Gerlich and Luer (65) and Angarano et al (3) describe a modification of the CORAB assay for the determination of anti-HBc IgM. However, capture assays for detecting anti-HBc IgM (76) have become more widely used for the diagnosis of acute HBV infection.

Assays for HBeAg and anti-HBe : Commercial RIAs for detecting HBeAg and anti-HBe were first produced by Abbott Laboratories in the 1980's (122) but have been largely replaced by a range of commercial ELISAs detecting either or both HBeAg and anti-HBe. To detect HBeAg in a serum sample a "sandwich" procedure may be used. Test serum is added to the anti-HBe solid phase followed by labelled anti-HBe, the binding of which is then measured. Anti-HBe assays use a neutralization principle where the test sample is mixed with an equal volume of HBeAg of precalibrated potency, which is neutralized by the presence of anti-HBe in the test sample. A "sandwich" method is then used to determine the presence (= anti-HBe negative) or absence (= anti-HBe positive) of HBeAg in the mixture.

SALIVA AS A DIAGNOSTIC FLUID

Most body fluids contain antibody but at a generally much lower concentration than is found in blood. This has limited their use as diagnostic specimens for viral antibody tests. It is well known that secretory IgA (sIgA) antibody is found in saliva but it is now recognised that other classes of immunoglobulin are able to gain entry into the oral cavity and are present in saliva at concentrations which, whilst being lower, do reflect those found in plasma (119, 120, 139).

Saliva is a mixture of several components including immunoglobulins (Table 3). Salivary antibodies come from two sources. The majority of antibody of diagnostic importance is present as a transudate from the capillary bed beneath the margin that separates the teeth and gum known as the gingival crevice. The antibody content of this crevicular fluid closely reflects the immunoglobulin class and specificities of antibody found in plasma. It contains both IgG and IgM with the same specificities as those antibodies already present in plasma (120, 127).

Table 3. The mean concentrations (mg/L) of immunoglobulin components of saliva (120).

Specimen	IgG	IgM	IgA
Plasma	14730	1280	2860
Parotid saliva	0.36	0.43	39.5
Crevicular Fluid	3500	250	1110
Whole saliva	14.4	2.1	19.4

The development of sensitive "antibody capture" assays enabled saliva to be used as the basis for successful and reliable diagnosic antibody tests. Antibody capture assays were first described by Flehmig et al (54) and Duermeyer et al (49) in the late 1970s

and are now extensively used in the diagnosis of virus infection. Capture formats are based upon the principle that a particular class of human immunoglobulin (IgG, IgM or IgA) is "captured" or bound by an antiserum to that immunogloblin previously fixed to a solid phase such as a polystyrene bead or the well of a microtitre plate. This solid phase can then be probed for the desired antibody specificity by the addition of a specific viral antigen, followed by the addition of a detector system. This commonly comprises a mouse monoclonal antibody, specific for the virus antigen under test, to which a ^{125}I labelled (RIA) or enzyme (e.g. horse raddish peroxidase) conjugated antibody (ELISA) is then directed. Signals generated using an RIA format are measured in a gamma counter. In the case of ELISA they are measured colourimetrically by a plate reader after the addition of a chromogenic substrate, such as tetra methyl benzidine (TMB).

When considering antibody capture assays it is important to recognise that the size of signal generated reflects the ***proportion*** of the total immunoglobulin of the class being captured that is specific for the antigen under test rather than the ***concentration*** of the specific antibody. Since saliva is composed partly of plasma transudate, the proportion of specific to total immunoglobulin will be similar in both serum and saliva; signals generated by such salivary assays can thus be considered independent of immunoglobulin concentration and therefore representative of serum (120, 127, 139). Moreover, in antibody capture assays, very low concentrations of IgG or IgM antibody are required to saturate the solid phase and they can therefore be successfully used with body fluids other than serum, such as saliva or even urine (32, 119, 139).

For large epidemiological studies collecting blood can be difficult, particularly for those populations outside the clinical environment and those disliking the invasive nature of venepuncture (119, 128). Saliva collection represents a non-invasive alternative to serum collection with clear advantages (Table 4) over venepuncture. Saliva collection is convenient, cheap and does not require the participation of trained personnel. It is also painless and less hazardous than venepuncture thus giving better access to large populations, hard to reach groups and children (119, 128, 139, 186).

Table 4. The potential advantages of saliva over serum specimens (139)

Advantage	Reason
More Acceptable	Noninvasive; painless; fewer refusals
More Convenient	self-collected; cheap; rapid; simple; no sterile precautions
More Versatile	reaches difficult populations e.g. IVDUs, isolated rural communities

The means by which saliva specimens are collected is an important practical consideration if saliva is to be used as a reliable alternative to serum collection for antibody testing. Since crevicular fluid contains most of the diagnostically important plasma-derived IgG and IgM antibodies it seems logical that crevicular fluid is well represented in the sample collected and is diluted as little as possible by the presence of other components of oral fluid during the collection process (139).

Collection devices

Saliva specimens are easily collected and several commercially available devices have been developed specifically for this purpose (Fig. 2) (119, 139). The devices used for this study include the Oracol (previously known as the Foam Swab) (19) (Malvern

Medical Developments, Worcester, UK), Omnisal (Saliva Diagnostics Systems Ltd, Singapore) and OraSure (Epitope Inc, Beaverton, UK) (Table 5) and are shown in Fig. 2. They all collect saliva using absorbent material placed in the mouth from which oral fluid can be eluted at a later date and have been demonstrated to yield samples that give a better quality of result compared to collecting by simply dribbling into a specimen container (124). The Foam swab and OraSure differ from the Omnisal device in that they are designed to be rubbed along the gums and so directly target the crevicular fluid. Samples collected by these absorbtive devices are correctly termed ***oral fluid*** (185). They should be distinguished from saliva secreted from glands discharging into the oral cavity which requires different devices for collection. For the remainder of this thesis, therefore, the term ***oral fluid*** is used.

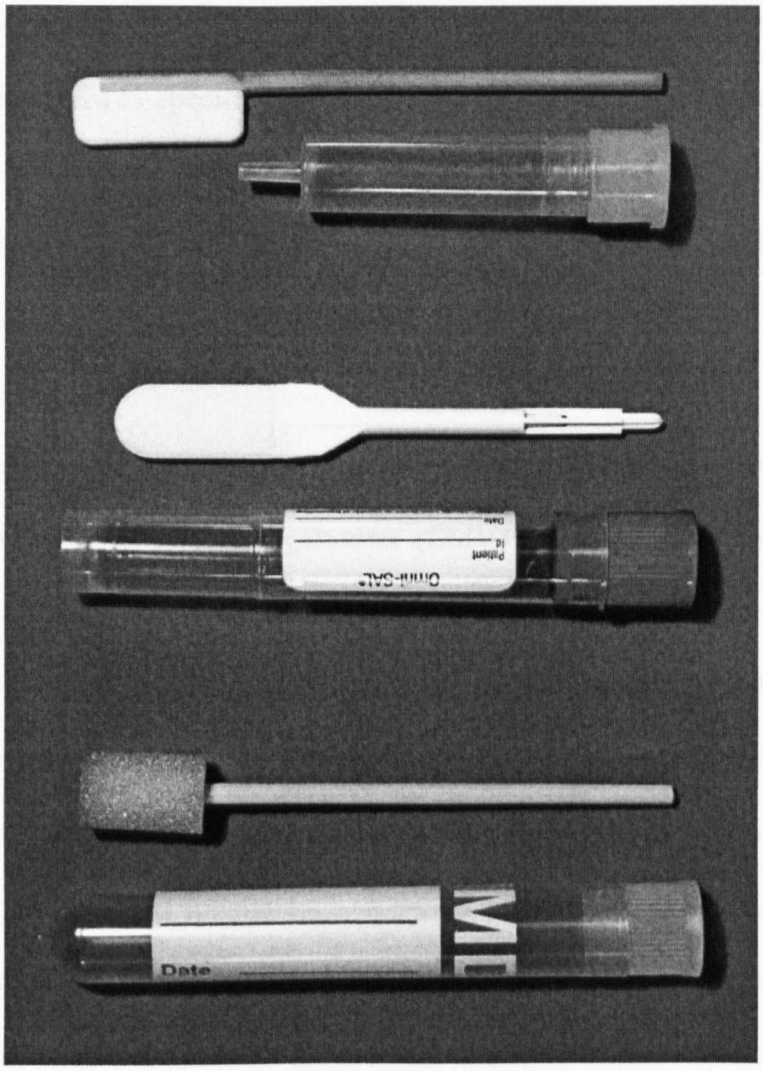
Table 5. A comparison of the features of three saliva collection devices (121)

Feature	Oracol	Omnisal	OraSure
stability (room temperature)	5 days	> 5 days	21 days
lab manipulation	add buffer, recover eluate	compress filters	centrifuge
Total IgG quantification	essential	desirable	essential
unit cost*	£0.50	£1.80	£2.50

*current costs as of March, 2000

Figure 2: Saliva collection devices

(From top to bottom: OraSure, Omni-SAL and Oracol)



Oral fluid IgG and IgM capture assays have been developed against a range of viral antigens and lead to the realisation that oral fluid testing could have broad applications in viral diagnosis, surveillance, outbreak investigation and vaccine monitoring (64, 139). Initially many of these tests used radioimmunoassay technology to provide the desired level of sensitivity and specificity but several, particularly assays that detect IgG specific for human immunodeficiency virus (HIV) surveillance, have now become available as commercial ELISA tests (74, 82, 139).

Anti-HIV assays are amongst the most widely used oral fluid assays and their use has been well described and evaluated (31, 74, 82, 101, 105, 140, 178). Other oral fluid assays that detect virus specific antibodies include IgG and IgM specific antibody capture radioimmunoassays for measles, mumps and rubella (144), parvovirus B19 (40, 157), hepatitis A and B (141), and an IgG specific antibody capture radioimmunoassay for Epstein Barr virus (EBV) (39, 186).

Assays described using formats other than antibody capture include an indirect ELISA to detect oral fluid IgG to cytomegalovirus (CMV) (187), an ultrasensitive EIA for detecting hepatitis A virus-specific IgG (131) and a commercial EIA for total antibody to hepatitis C virus (HCV), adapted for use with oral fluid samples (180). Commercial EIA assays designed for use with serum have also been adapted for use with oral fluid samples by modifcation of the protocol for the detection of HCV antibodies (10, 20) and measles, mumps and rubella IgG (179). In addition to the detection of virus specific antibodies in oral fluid, commercially available EIA assays designed for use with serum have also been adapted for use with oral fluid for the detection of specific IgG antibody against the human stomach bacterial pathogen Helicobacter pylori (142, 171).

Oral fluid assays for rubella and HBc specific IgG.

Assays have been described which are designed to detetct rubella specific IgG and HBc specific IgG in oral fluids (139-141). Perry et al (144) described an IgG antibody capture radioimmunoassay (GACRIA) for rubella specific IgG which has subsequently been used for epidemiological surveys (51, 124). A prototype rubella specific IgG antibody capture ELISA (GACELISA) was described by Parry et al

(140) but required further refinement and evaluation to supersede the radioimmunoassay. Parry et al (140) also described a radioimmunoassay for anti-HBc IgG in saliva samples which was adapted to an ELISA format though was found to be less sensitive when testing weakly seropositive subjects. Murex have since developed an EIA (ICE Hbc Detection Pack) which detects both IgG and IgM to the core protein of HBV. This assay has been adapted for use with oral fluid samples and has subsequently replaced the IgG capture radioimmunoassay for HBV described by Parry et al (141).

The requirement for simpler oral fluid assays

Population-based surveys of antibodies to both viral and bacterial antibodies have important and well established uses in immunity surveillance and epidemiological research, especially when assessing the impact of vaccination and identifying target groups for vaccination. Where community-based studies of antibody prevalence are required the collection of oral fluid samples has clear practical advantages providing suitable assays are available (150, 151). Currently many of the assays available for testing oral fluids are only available in a radioimmunoassay format and, whilst they are sensitive and well characterised, they do have several disadvantages. Radioimmunoassay techniques are often time consuming, technically demanding, expensive, require special equipment (e.g. gamma counter), are not easily transferable between laboratories and produce radioactive waste. Therefore there is a clear need for the development of simple, sensitive and robust ELISA assays which produce less hazardous waste products and are suitable for field use and in basic laboratories, especially in developing countries. Here the requirement for identification of target groups for vaccination is an essential element of disease control programmes (42).

MATHEMATICAL MODELLING

The theoretical basis by which communicable diseases are transmitted has been well understood since the beginning of the 20th century and has been described using mathematical models. The mathematical modelling of the transmission of infectious diseases aims to describe the various stages of a disease through which an individual may pass, from susceptibility through to immunity, and the associated factors that may determine the transmission from an infected to a susceptible individual. Perhaps the greatest challenge faced by the mathematical modeller is trying to define and represent those factors which can be used to precisley define a contact between a susceptible and infectious individual and is especially difficult in the case of respiratory transmitted diseases (112, 160).

The accuracy of mathematical models is able to be tested using epidemiological data obtained from community based studies. Mathematical models can be used to help design vaccination programmes and predict the impact they are likely to have. However, it is only with the advances made in computer science over the last two decades that sufficient computing power has been available to implement simulations sufficiently large and detailed to make practical predictions possible. Developments made in the use of oral fluids as an alternative to serum sampling are of great interest to mathematical modelers since they should greatly facilitate the ease and accuracy with which these models can be tested in order to gain a true insight into the effect present or future vaccination programmes may have.

OBJECTIVES OF THE STUDY

1. Technical development of oral fluid antibody assays

1.1. Evaluation of oral fluid collection devices

1.2. Development of ELISA for detecting rubella IgG in oral fluid

1.3. Development of ELISA for the detection of anti-HBc in oral fluid

1.4. The use of these ELISA assays in field studies

2. Rubella molecular epidemiology

2.1. The development and optimisation of a rubella PCR suitable for both diagnosis and molecular epidemiology

2.2. The use of PCR to investigate the phylogeny of rubella

2.3. The extent to which oral fluid may be used as a clinical specimen for the detection of virus genome

CHAPTER 1

EVALUATION OF ORAL FLUID COLLECTION DEVICES

OBJECTIVES

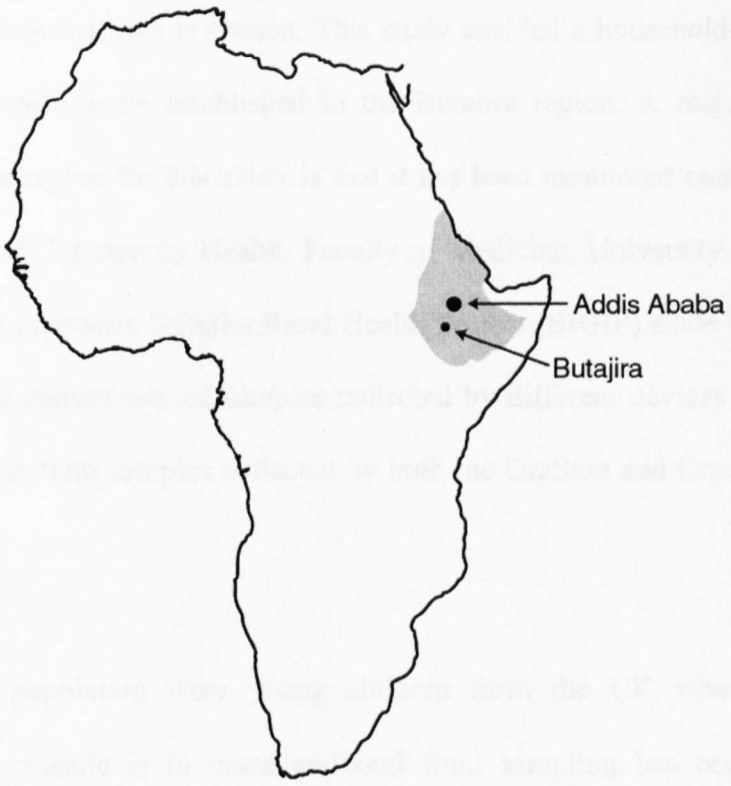
There were three main objectives for this part of the study:

- to demonstrate the utility of oral fluid collection in epidemiological studies.
- to compare three oral fluid collection devices (Oracol, OraSure and Omnisal) in terms of the quality of oral fluid collected.
- to determine the collection device most suitable for viral specific IgG antibody screening.

Introduction

The collection devices were evaluated in two contrasting populations. The first was from the rural district of Butajira in south western Ethiopia (Fig. 3). This region is approximately 130 km south of Addis Ababa. The population is approximately 250,000 and consists predominantly of subsistence farmers. There is currently no HBV or rubella immunization and EPI vaccination coverage for measles varies considerably and is related to distance from the town of Butajira. This study involved subjects aged <5 years to ≥50 years.

Figure 3: A map showing the Butajira region of Ethiopia



The second population were children aged 3.5 - 5 years living in north Hertfordshire, UK. All had recently received MMR vaccine as part of a "pre-school boost" (PSB) study to assess the response to a second dose. These are therefore two populations in which the epidemiological study of certain vaccine preventable virus infections was important and represented markedly different epidemiological and vaccination contexts.

The first study population, covering a wide age spectrum, is from a rural region in a developing country where vaccination coverage is poor and limited to a few infectious diseases. In order to extend vaccination coverage for other vaccine preventable

infections in this population, it is important that their epidemiology is fully understood. If oral fluid samples are to be usedfor this purpose, it is important that the optimal collection device is chosen. This study enabled a household-based oral fluid collection system to be established in the Butajira region. A major reason in the choice of this region for this study is that it has been monitored continuously by the Department of Community Health, Faculty of Medicine, University of Addis Ababa as part of the long-term Butajira Rural Health Project (BRHP) since 1986 (128, 168). To assess the consistency of samples collected by different devices a representative number of oral fluid samples collected by both the OraSure and Oracol devices were obtained.

The second population were young children from the UK where an extensive vaccination schedule is in place and oral fluid sampling has been used for the surveillance of measles, mumps and rubella (MMR) since 1994. Whilst there are a variety of methods now available for collecting oral fluid, there have been few studies investigating which is optimal for community use and none exclusively involving young children. This has permitted the effect of the childhood MMR vaccination programme to be monitored. MMR vaccine was introduced in 1988 as a single dose at 12-15 months, supplemented by the MR campaign in 5 to 15 year old children in 1994 and more recently, in 1998, was augmented by a two dose schedule, with a second dose being offered to pre-school children aged 3.5 to 4 years (62, 63, 111). It may be that the optimal device for each of the populations used in this study are not necessarily the same.

The part of the study involving children from the UK was performed by collecting oral fluid samples using all three devices plus a matching serum from each individual. Total IgG and IgM antibody concentrations of the oral fluid collected by each device were measured and the rubella specific IgG concentration of oral fluid compared to that of the matching serum sample as measured by a sensitive commercially available ELISA kit. Serum and matching oral fluid samples from the children involved in the PSB study were also screened for parvovirus B19 specific IgG. All parvovirus B19 serology was carried out by the Immunisation and Diagnosis Unit, ERNVL, CPHL and the results made available by Dr BJ Cohen. As a result of vaccination a high proportion of the population of children in this study were expected to have antibody to rubella (133). By contrast, the antibody prevalence to parvovirus B19 is low amongst this age group (27) enabling an assessment of the specificity of oral fluid testing to be made. In investigating ***acceptability*** of each collection device to study participants, ease of administration and comfort were assessed by questionnaire. Results of the questionnaire were analysed by Dr Mary Ramsay of the Immunisation Division, PHLS Communicable Disease Surveillance Centre, London.

MATERIALS AND METHODS

Evaluation of oral fluid collection devices in rural Ethiopia

Oral fluid collection and extraction.

Oral fluid was collected and extracted from sterile foam swabs ('Oracol', Malvern Medical Developments, Worcester, UK) as previously described (19, 186) and from the OraSure (Epitope Inc, Beaverton, UK) and Omnisal (Oral fluid Diagnostics Systems Ltd, Singapore) devices according to the manufacturers instructions. All samples were stored at -20^{0}C until required for testing.

The Oracol device consists of an expanded polystyrene foam swab fixed to a plastic stick (Fig.2). It was used to collect oral fluid by rubbing the swab along the sides of the gums, both inside and out, for one minute which was then placed into an accompanying tube. To extract the oral fluid 1 ml of transport medium (Phosphate Buffered Saline [PBS] containing 10% fetal calf serum [FCS], 0.2% Tween 20, 0.5% Gentamicin 50mg/ml stock, 0.2% Fungizone 250ug/ml stock) was added to the tube containing the swab and thoroughly mixed. The swab was then inverted, returned to the tube, and centifuged at 2000rpm for 5 min. The swab was then removed and discarded and the fluid at the bottom of the tube pipetted into a storage vial.

The OraSure device was used to collect oral fluid by gently moving the absorbent pad along the gum twice and then holding it stationary against the lower gum on one side for 2 minutes. To extract oral fluid the small plastic tip at the base of the OraSure tube was broken off and discarded and the remainder of the tube containing the used absorbent pad centrifuged at 2000rpm for 5 min. The oral fluid was then pipetted and stored.

The Omnisal was used to collect oral fluid by placing and keeping the absorbent swab under the tongue until the stem indicator of the device turned blue. The flat lint swab was then detached and placed in the accompanying tube into which an Omnisal filter plunger was also inserted and depressed to extract the oral fluid which was pipetted and stored. A summary of the method of use for each of the three collection devices is shown in Table 6.

Table 6. Methods for oral fluid collection by each device

Device	Description of use	Cost
OraSure	Move pad gently along gum twice. Place against lower gum on 1 side and keep stationary for 2 min	**£2.50**
Omni-SAL	Place pad under tongue and keep still until indicator of device turns blue	**£1.80**
Oracol	Rub sponge firmly along the base of the gums (inside and out) of the upper and lower jaw, for 1 min, using an action similar to tooth brushing	**£0.50**

Serum collection.

Venous blood was collected in the home from each participating individual by medical personnel from the Butajira Health Clinic using the Vacutainer system (5ml Vacutainers and Green or Black needles, Becton Dickinson). Blood samples were allowed to clot for 2-3 hours at room temperature and serum then transferred into a 2ml screw-cap storage vial using a plastic pasteur pipette, and stored at -20°C.

Samples collected

Samples were obtained from 58 participants in 14 households from the village of Dirama, 2km from Butajira town. A serum sample, with matching oral fluid samples

of sufficient volume for all laboratory tests obtained using each of the three oral fluid collection devices, was provided by 38 individuals. A total of 13/38 individuals and 24/38 individuals provided duplicate oral fluid samples collected by the Oracol and OraSure devices. Individuals were asked to provide an oral fluid sample using a different device on each of three consecutive days in which collection took place, at approximately the same time during each day. The order of collection was randomised for each household. Blood was collected on the third day of the collection period (128).

Analysis of total antibody concentration.

Total IgG concentration (mg/L) in oral fluid samples was measured by ELISA as described by Connell et al (32). The method is detailed in Appendix 1.1 (***pp. 80-82***). This method was adapted to measure the total IgM concentration (mg/L) in oral fluid (Appendix 1.1, pp. 80-82).

Measurement of rubella specific IgG antibody.

The measurement of rubella specific IgG in oral fluid samples was carried out by radioimmunoassay as described by Perry et al (144) and is described in detail in Appendix 1.2 (***pp. 83-84***).

The concentration of rubella specific IgG in matching serum samples was measured using a commercial ELISA kit (Behring Enzygnost, Behringwerke AG, Marburg, Germany) which is described in detail in Appendix 1.3 (***pp. 85-87***).

Statistical Methods.

To assess the quality of oral fluid collected by each make of device three methods were used.

1. **Geometric mean (GM).** Mean concentrations of total IgG and total IgM, with 95% confidence intervals, were calculated using the geometric mean [$\log_{10}$ (mg/L)] (5).
2. **Spearman's rank correlation (r).** The degree of association between the rubella specific IgG concentration in serum and matching oral fluid was calculated, with 95% confidence intervals, for each of the three collection devices using Spearman's rank correlation (5).
3. **Single factor analysis of varience (ANOVA).** The hypothesis that the mean logarithm of total IgG and IgM antibody concentrations for oral fluid samples collected by each device are the same was tested using single factor ANOVA (5).

Evaluation of oral fluid collection devices amongst children aged 3.5 - 5 years in north Hertfordshire, UK.

Survey Details

One hundred and forty three children aged 3.5-5 years living in north Hertfordshire, UK, participated. All had recently received MMR vaccine as part of a pre-school boost (PSB) study primarily designed to assess the response to DTaP vaccine also given at this age. The children were randomised to use one of the three types of oral fluid collection devices being assessed, namely Orasure, Omni-SAL and Oracol (Table 1). A parent used the device to obtain an oral fluid sample from the child, and completed a short questionnaire recording information on ease of use and willingness

to use the device again. Parents were asked to respond using a Likert scale which scored the acceptability of use of each device on a scale of 1 - 5. General comments regarding the use of each of the devices were also recorded.

Sample collection and processing

Instructions for oral fluid collection were supplied on the packaging by the manufacturer (Table 6). After collection of oral fluid, devices were placed into the appropriate transport container and labelled with a unique identification number. Collected samples were then transported by post to the Enteric and Respiratory Virus Laboratory (ERVL), CPHL, where the oral fluid was extracted according to the manufacturers' instruction for OraSure and Omni-SAL and as previously described for the Oracol (19) and stored in labelled screw capped microtubes at -20^{0}C until required for testing.

Venous blood samples were collected by nursing personnel using a Vacutainer System. Bloods were allowed to clot and serum aspirated into labelled screw capped microtubes and stored at -20^{0}C until required for testing.

Laboratory Methods

Serum samples were screened for rubella specific IgG by ELISA (Rubella IgG Enzygnost, Behring Diagnostics, Behringwerke AG, Marburg, Germany) according to the manufacturer's instructions as previously described, with the results expressed as International Units (IU)/ml (Appendix 1.3). Serum samples were screened for parvovirus B19 specific IgG using ELISA (Dako, Ely, UK) and a detailed method is described in Appendix 1.5 ***(pp. 99-92)***

Oral fluid samples were screened for rubella specific IgG by IgG capture radioimmunoassay as previously described with results expressed as the test sample to negative (T/N) ratio (144). Oral fluid samples were screened for parvovirus B19 specific IgG by IgG antibody capture radioimmunoassay (GACRIA) as described by Rice and Cohen (1996) (157). A detailed method is described in Appendix 1.4 (***pp. 87-88***).

The total IgG and total IgM content of oral fluid samples was measured using an indirect ELISA assay as previously described (32) (Appendix 1.1).

Statistical Methods.

The quality of oral fluid collected by each make of device and acceptability of each to participants was evaluated by four methods.

1. **Geometric mean (GM).** GM concentrations of total IgG and total IgM, with 95% confidence intervals, were calculated using the geometric mean [$\log_{10}$ (mg/L)] (5).
2. **Spearman's rank correlation (r).** The degree of association between the rubella specific IgG result in serum and matching oral fluid for each of the three collection devices was calculated, with 95% confidence intervals, using spearman's rank correlation (5).
3. **Chi-squared Fisher's exact test.** In assessing the acceptability of each device the proportion of parents responding to each question was compared using Chi-squared Fisher's exact test as appropriate (5).
4. **Single factor analysis of varience (ANOVA).** The hypothesis that the mean logarithm total IgG and IgM antibody concentrations for oral fluid samples collected by each device are the same was tested using single factor ANOVA (5).

RESULTS

Evaluation of oral fluid collection devices in rural Ethiopia

A matching serum and oral fluid sample of sufficient volume for all required laboratory testing using each of the three collection devices was provided by 38 of the 58 persons who participated in the study. Of these, 13 persons provided duplicate oral fluid samples collected using the Oracol and 24 persons provided duplicate oral fluid samples collected using the OraSure device. A total of 34/38 (89.5%) had IgG antibody to rubella by serum ELISA.

Quality of sample

The total IgG and IgM concentrations of the oral fluid samples provided by each of the three collection devices is compared in Table 7.

Table 7. A comparison of immunoglobulins in oral fluid collected by each device (Ethiopian population)

	OraSure	Omni-SAL	Oracol
Number	38	38	38
Total IgG (mg/L):			
Geometric Mean	22.65	24.71	41.23
(95% CI)	(18.00 - 28.51)	(18.04 - 33.86)	(27.36 - 62.12)
Total IgM (mg/L):			
Geometric Mean	1.39	2.52	3.12
(95% CI)	(1.039 - 1.867)	(1.76 - 3.61)	(2.41 - 4.02)
Rank correlation (r_s): rubella specific IgG - serum vs oral fluid **(95% CI)**	0.434 (0.13 - 0.71)	0.604 (0.31 - 0.83)	0.565 (0.30 - 0.79)
Rubella specific IgG results in paired serum and oral fluid samples:			
overall agreement	28/38 (74%)	29/38 (76%)	29/38 (76%)

The Oracol device provided oral fluid samples containing the highest geometric mean (GM) concentrations of both total IgG and IgM, followed by the Omni-SAL, with the OraSure device yielding oral fluid samples with the lowest GM total IgG and IgM concentrations. Single factor ANOVA showed the difference between the three collection devices to be significant for the GM concentration of total IgG ($p = 0.026$) and total IgM ($p = 0.001$) obtained for each.

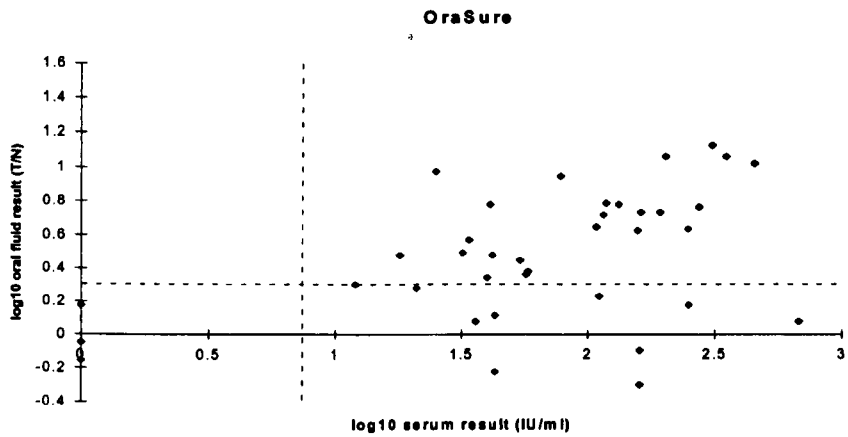
A comparison of the rubella specific IgG result in serum determined by ELISA with that in oral fluid collected by each device as determined by GACRIA is shown in Table 7. There is little difference between each device using rank correlation, though the Omni-SAL and Oracol devices provided marginally improved rank correlation with serum antibody levels over OraSure results. This is supported by the scatter graphs shown in Fig. 4 which compare the rubella specific IgG results in matching serum and oral fluid samples for each of the collection devices.

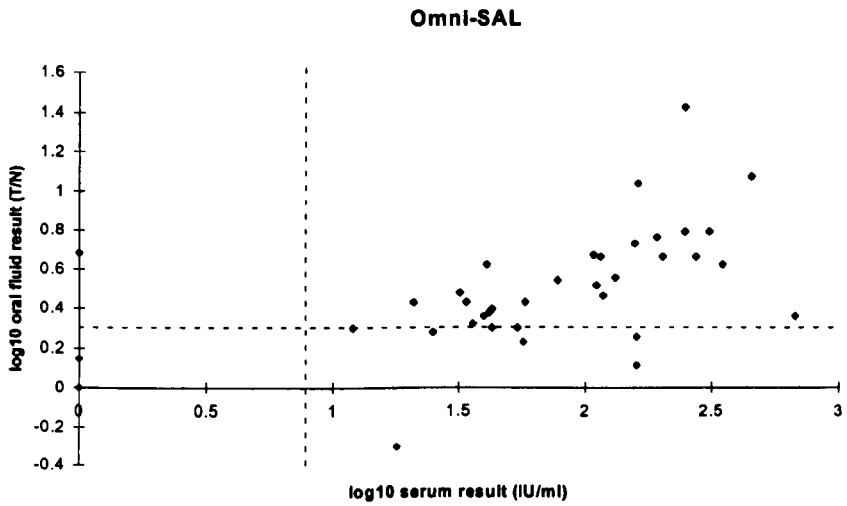
An assessment of the performance of oral fluid samples collected by each of the devices was also made by calculating the agreement between results by comparing those of the GACRIA to a commercial ELISA (Behring) which was used to screen corresponding serum samples for rubella specific IgG (Table 7). The agreement was very similar for each of the three devices, being 74%, 76% and 76% for the OraSure, Omni-SAL and Oracol respectively. It was not appropriate to estimate sensitivity and specificity values relative to the serum ELISA due to the small numbers of samples available for each of the collection devices. However, a 2x2 table showing how oral fluid results compared to matching serum results is shown in Table 8.

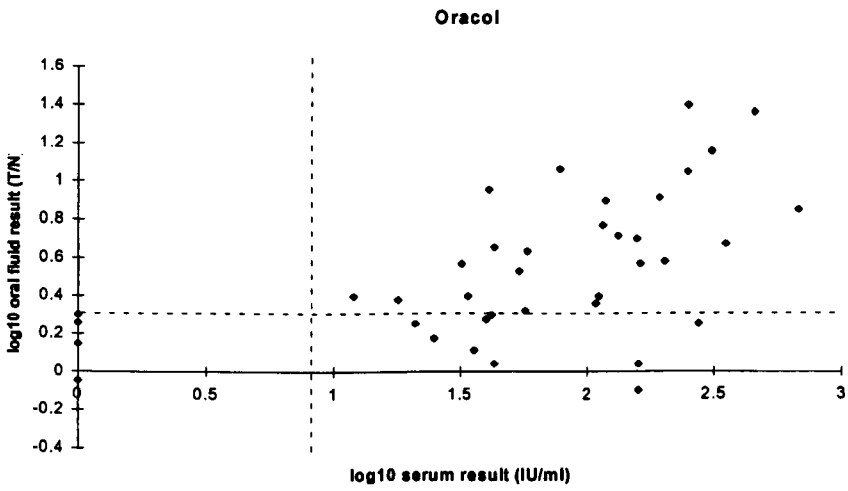
Table 8. Rubella specific IgG results in sera and corresponding oral fluids for each of the oral fluid collection devices used by the rural Ethiopian population.

ORAL FLUID result (GACRIA)		SERUM result (Behring)	
		POS	NEG
OraSure	POS	24	0
	NEG	10	4
Omni-SAL	POS	26	1
	NEG	8	3
Oracol	POS	25	0
	NEG	9	4

Fig. 4 A comparison of rubella specific IgG results in matching serum and oral fluid samples for each collection device (Ethiopian population) [broken lines represent assay cut off values]







Comparison of duplicate oral fluid samples collected by Oracol and Orasure

Total IgG and IgM concentration and rubella specific IgG concentration in duplicate oral fluid samples collected using the Oracol and Orasure devices were compared using rank correlation (Table 9).

Table 9. A comparison of duplicate oral fluid samples using rank correlation.

Category	Oracol (95%CI) [n = 13]	OraSure (95%CI) [n = 24]
Total IgG	r = 0.907 (0.77 - 0.98)	r = 0.471 (0.15 - 0.79)
Total IgM	r = 0.483 (-0.16 - 0.89)	r = 0.701 (0.38 - 0.89)
Rubella specific IgG	r = 0.940 (0.84 - 0.99)	r = 0.580 (0.16 - 0.88)

These data show that for total IgG concentration and rubella specific IgG concentration, correlation of results from duplicate samples of oral fluid collected by Oracol were considerably higher than for duplicate samples collected by the OraSure device. The correlation between duplicate samples for total IgM antibody was higher for the OraSure device than the Oracol, though the 95% confidence intervals were wide in all cases.

Acceptability of oral fluid collection devices

The acceptability to participants of each oral fluid collection device was assessed by Dr D J Nokes, Department of Biological Sciences, University of Warwick (128). Briefly, all participants found each make of device simple to use. The results are summarised in Table 10.

Table 10. Acceptability of oral fluid collection devices to participants from rural Ethiopia*

Comments	OraSure	Omni-SAL	Oracol
Uncomfortable to use	4/22 (18%)	11/43 (26%)	3/41 (7%)
Disliked taste (Bitter/salty/hot/dusty)	16/28 (57%)	2/28 (7%)	2/28 (7%)
No. children <10 years who chewed device	3/9 (33%)	5/9 (56%)	3/12 (25%)

* Includes all oral fluid samples collected regardless of whether a matching serum was also obtained.

Evaluation of oral fluid collection devices amongst 3.5 - 5 yr old children living in north Hertfordshire

A matching serum and oral fluid sample of sufficient volume for all required laboratory testing was provided and the type of oral fluid collection device used recorded for 126 of the 143 children in this study. By serum ELISA 126/126 (100%) had rubella IgG and 13/126 (9.7%) had parvovirus B19 IgG.

Quality of sample

The total IgG and IgM concentrations of the oral fluid samples provided by each of the three collection devices is summarised in Table 11.

The Oracol device provided oral fluid samples containing the highest geometric mean (GM) concentrations of both total IgG and IgM, followed by the OraSure, with the Omni-SAL device yielding oral fluid samples with the lowest GM total IgG and IgM concentrations. Single factor ANOVA showed the difference between the three collection devices to be significant for the GM concentration of total IgG ($p < 0.0001$) and total IgM ($p = 0.03$) obtained for each.

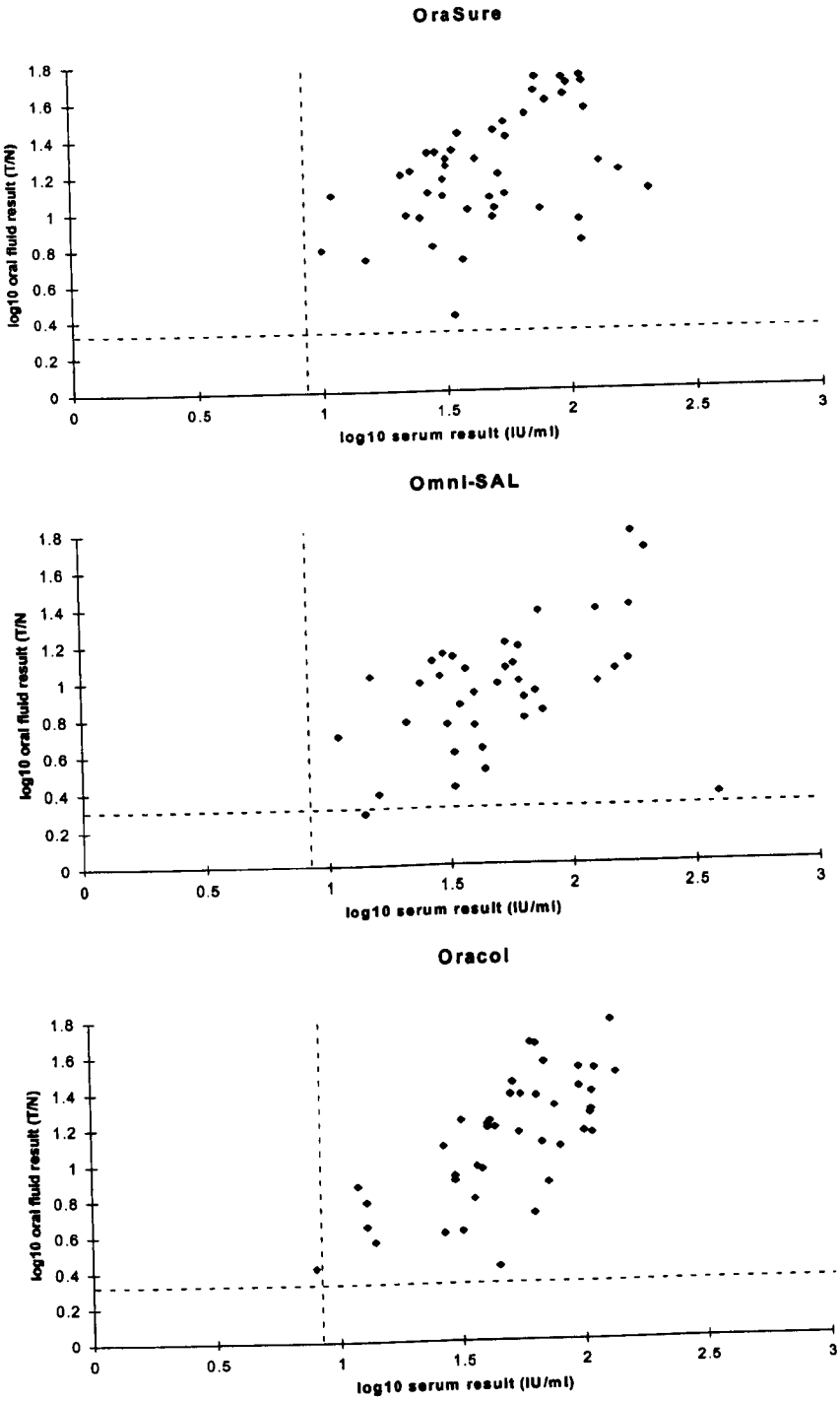
A comparison of the rubella specific IgG result in serum determined by ELISA with that in oral fluid for each collection device as determined by GACRIA is shown in Table 11. Rubella specific IgG T/N values in oral fluid and IU/ml in serum correlated most closely when oral fluids were collected by Oracol, followed by Omni-SAL samples with the poorest correlation obtained with oral fluids collected by OraSure.

Table 11. An assessment of the oral fluid quality collected by three collection devices

	OraSure	Omni-SAL	Oracol
Number	45	38	42
Total IgG (mg/L):			
Geometric Mean	4.40	1.99	6.08
(95%CI)	(3.56 - 5.43)	(1.60 - 2.47)	(4.69 - 7.88)
Total IgM (mg/L):			
Geometric Mean	0.79	0.90	1.39
(95%CI)	(0.61 - 1.02)	(0.62 - 1.32)	(1.01 - 1.90)
Rank correlation (r_s):			
rubella specific IgG - serum vs oral fluid (95% CI)	0.454 (0.22 - 0.66)	0.647 (0.40 - 0.82)	0.742 (0.61 - 0.85)
Rubella specific IgG results in paired serum and oral fluid samples:			
Overall agreement	44/45 (98%)	36/39 (92%)	41/42 (98%)
Mean ratio of rubella specific IgG result in serum (Behring ELISA, IU/ml) to that in matching oral fluid (GACRIA, T/N) (95% CI)	2.9 (2.3 - 3.6)	5.8 (4.4 - 7.6)	3.7 (3.1 - 4.4)
B19 specific IgG results in paired serum and oral fluid samples:			
Overall agreement	41/45 (91%)	38/38 (100%)	41/42 (98%)

The scatter graphs shown in Fig. 5 show a comparison of rubella specific IgG results in matching serum and oral fluid samples for each of the collection devices. These suggest that there is little to discriminate between the three devices when comparing the rubella specific IgG in oral fluid samples to that in serum. The mean ratio of rubella specific IgG concentration in sera (IU/ml) to that of matching oral fluids (T/N) collected by each make of device was highest for those samples collected by Omni-SAL (Table 11). This suggests that of oral fluid samples collected by each of the three devices, the rubella specific IgG result (T/N) measured in those collected by Omni-SAL compares least favourably to that measured in the matching serum by ELISA (IU/ml).

Fig. 5 A comparison of rubella specific IgG results in matching serum and oral fluid samples for each collection device (children, north Herts, UK) [broken lines represent assay cut off values]



An assessment of the performance of oral fluid samples in virus specific antibody assays was made by calculating the agreement between results by comparing those of the GACRIA to a commercial ELISA which was used to screen corresponding serum samples. A similar high agreement (>90%) was seen for each of the three devices for both rubella specific IgG and parvovirus B19 results when the oral fluid and serum results were compared (Table 11). Since 100% and 9.7% of serum samples were demonstrated to contain rubella specific IgG and parvovirus B19 specific IgG respectively this ***suggests*** that the sensitivity and specificity was high for each of the three devices. However, it was not appropriate to estimate actual sensitivity and specificity values relative to serum ELISA due to the small numbers of samples available for each of the collection devices. A 2x2 table showing how oral fluid results compared to matching serum results is shown in Table 12.

Table 12. Rubella and parvovirus B19 specific IgG results for sera and corresponding oral fluids for each of the oral fluid collection devices used by the population of children from north Hertfordshire.

SPECIFIC IgG	ORAL FLUID result (GACRIA)		SERUM result (ELISA)	
			POS	NEG
Rubella	OraSure	POS	44	0
		NEG	1	0
	Omni-SAL	POS	36	0
		NEG	2	0
	Oracol	POS	41	0
		NEG	1	0
Parvovirus B19	OraSure	POS	0	37
		NEG	4	4
	Omni-SAL	POS	0	34
		NEG	4	0
	Oracol	POS	0	37
		NEG	4	1

Acceptability of oral fluid collection devices.

The proportion of parents answering that they found the oral fluid collection device either quite easy or very easy to use was 45/47 (96%) for both the Oracol and the OraSure, higher than for the Omni-SAL (20/37 [54%], $p<0.001$). The proportion who would probably or definitely not take another "saliva" test was higher for the Omni-SAL (6/37 [16%]) than for the Oracol (0/47 [0%], $p = 0.006$) and the OraSure (2/47 [4%], $p=0.07$). These results are summarised in Table 13.

Table 13. Acceptability of oral fluid collection devices to children from north Hertfordshire, UK.

Comments	OraSure	Omni-SAL	Oracol
Quite/very easy to use	45/47 (96%)	20/37 (54%)	45/47 (96%)
Probably/definitely not take another "saliva" test	2/47 (4%)	6/37 (16%)	0/47 (0%)

Minor comments were offered in relation to all devices, mainly about the size and appearance of the swabs. For the OraSure and the Omni-SAL additional comments related to difficulty because of the the length of time that the child was required to keep the collection device in the mouth. One parent, who used the Omni-SAL, commented how much easier it was than vene-puncture.

A comparison of results from the rural Ethiopian population and children from north Hertfordshire

A comparison of results obtained from samples collected from the two populations used in this study is shown in Table 14.

Table 14 . A comparison of results from the rural Ethiopian population and children from north Hertfordshire

LOCATION	Rural Ethiopia	North Herts (UK)
Age range (yrs)	< 5 - ≥ 50	3.5 - 5
No. TESTED		
• OraSure	38	45
• Omni-SAL	38	38
• Oracol	38	42
Total IgG (mg/L) [95% CI]		
• OraSure	22.7 [18.0-28.5]	4.4 [3.6-5.4]
• Omni-SAL	24.7 [18.0-33.9]	2.0 [1.6-2.5]
• Oracol	41.2 [27.4-62.1]	6.1 [4.7-7.9]
Total IgM (mg/L) [95% CI]		
• OraSure	1.4 [1.0-1.9]	0.8 [0.6-1.0]
• Omni-SAL	2.5 [1.8-3.6]	0.9 [0.6-1.3]
• Oracol	3.1 [2.4-4.0]	1.4 [1.0-1.9]
Rank correlation (rs): rubella specific IgG - serum vs oral fluid (95%CI)		
• OraSure	0.434 (0.13 - 0.71)	0.454 (0.22 - 0.66)
• Omni-SAL	0.604 (0.31 - 0.83)	0.647 (0.40 - 0.82)
• Oracol	0.565 (0.30 - 0.79)	0.742 (0.61 - 0.85)
Agreement: Rubella specific IgG results in paired serum and oral fluid samples		
• OraSure	74%	98%
• Omni-SAL	76%	92%
• Oracol	76%	98%

DISCUSSION

The following will be discussed:

- the quality of oral fluid obtained using each collection device and its suitability for antibody testing
- the consistency of oral fluid samples by a comparison of duplicate samples from the same individual
- the acceptability of each collection device to the participants

Results from each of the two populations that provided oral fluid samples will be compared and contrasted before conclusions are drawn about which collection device is optimal. Whilst data from the two populations was analysed in a similar way, two aspects of the study design were different for the two populations. These were taken into consideration when comparing results and drawing conclusions:

- The population from rural Ethiopia consisted of persons aged <5 years to ≥50 years of age, whilst the population from north Hertfordshire was made up entirely of children aged 3.5-5 years.
- Each of the 38 individuals from rural Ethiopia used in the analysis provided an oral fluid collected using each of the three devices being assessed. In contrast the children from north Hertfordshire were randomised to use one of the three types of oral fluid collection device being assessed.

Therefore a more strictly accurate comparison of the quality of oral fluid collected by each device being assessed can be made using data from the Ethiopian population, since the same individual provided an oral fluid sample collected using all three

devices. Also, the effect age may have on results was considered using samples provided by the Ethiopian population since a wide age range is covered.

QUALITY OF SAMPLE

The quality of oral fluid sample collected by each device for antibody testing was assessed by measurement of total IgG and IgM concentration and by measurement of rubella specific IgG concentration in comparison to that in a matching serum sample.

Total IgG and IgM concentration

Measurements of both the total IgG and IgM concentration in oral fluid samples provided by both populations suggested that there was sufficient present for oral fluid samples to be useful in antibody studies, confirming the findings of previous studies (19, 31, 39, 40, 51, 70, 82, 105, 119, 120, 128, 129, 139-141, 150, 151, 157, 178, 186, 187). A significant difference between the three devices was found when total IgG and IgM geometric mean (GM) concentrations were ompared. the patterns were similar for samples from both the rural Ethiopian population and the children from north Hertfordshire. Both the total IgG and IgM GM concentrations in oral fluid samples collected by the Oracol were higher than for those collected by OraSure and Omni-SAL (Table 7). This observation may be accounted for by the way in which the devices are used, though it is possible that the efficiency of antibody elution from absorbent material may vary between devices. The Oracol is designed to target the gums, the part of the oral cavity most likely to be rich in crevicular fluid, and is used vigorously. The OraSure, which also targets the gums, is used more passively than the Oracol which may account for the lower total antibody concentrations found in oral fluid samples collected by this device. The Omni-SAL, which is designed to be placed

under the tongue and is also used passively, provided oral fluid with a similar total IgG and IgM concentration to that of the OraSure device.

The suggestion that oral fluid collection devices which target crevicular fluid and are designed to be used vigorously provide a higher quality of sample for antibody testing and is supported to some degree by an earlier study by Parry et al (139). They (139) compared OraSure and Salivette, a device consisting of a cotton wool wad which is chewed to collect oral fluid from all parts of the oral cavity rather than crevicular fluid specifically. OraSure yielded oral fluid samples with a higher mean total IgG concentration than Salivette, consistent with the results of our study in that collection devices which target areas of the mouth rich in crevicular fluid provide a better quality of sample for antibody testing.

An interesting observation is that in the Ethiopian study population, the total IgG GM concentration was approximately seven times higher than that obtained in samples from children from north Hertfordshire, UK. This difference may be due to the age of the two study populations, as the Ethiopian population covered a broad age range and included a large proportion of adults, whereas the subjects from north Hertfordshire were all children aged between 3.5 and 5 years. The results may therefore reflect the developmental stage or maturity of the immune systems of the participants.

The different results in the two studies may also reflect socio-economic status which result in a higher exposure to infectious diseases and therefore higher antibody levels in developing countries. This is also consistent with the higher total IgM concentration found in subjects from rural Ethiopia. Being persons from a developing

country they are more likely to have been recently exposed to an infectious agent than their counterparts in the developed world. Ethnic origin is a further factor which may effect the immunoglobulin concentrations in oral fluid. Susceptibility to infectious disease and the extent to which a humoral immune response occurs is genetically determined and may vary between ethnic groups (46).

In a recent study by Wilson et al (193) using oral fluid from 121 school children from the UK (age range 7-11 years, mean age 8.0 years) collected by Salivette the total IgG concentrations were found to range from 3.8 mg/L to 165 mg/L. These data appear to correspond more closely to that from the Ethiopian population than children from north Hertfordshire. This is consistent with total antibody levels detected in oral fluid being related to the maturity and state of development of the subjects immune system.

A further factor that may have influenced the levels of total antibody found in oral fluid samples from the two populations is how samples were stored until the oral fluid could be extracted from the device and frozen at -20^{0}C in the laboratory. In Ethiopia, transport medium was added to Oracol samples on the same morning of collection and all samples stored at 2-10^{0}C until processed by the laboratory. In contrast, samples collected in the UK were kept at room temperature until processed and transport medium added when they were received in the laboratory for processing. There is a possibility therefore that the immunoglobulin content of those oral fluid samples collected in the UK may have deteriorated at a faster rate than in those collected in Ethiopia which is consistent with the considerably lower total immunoglobulin content, particularly IgG, found in samples from UK subjects.

Rubella specific IgG

Figures 4 and 5 show that the concentration of rubella specific IgG in oral fluid as measured by GACRIA was lower and varied more than that in matching sera as measured by ELISA. The oral fluid test may therefore be used reliably to give a ***qualitative*** result only.

Obtaining a ***quantitative*** result from oral fluid in the manner that is currently possible for serum with ELISA assays (e.g. Behring) represents a considerable challenge that may never be successfully met with current technology. There are a number of variable factors that must be considered when collecting and testing oral fluid samples which make it extremely difficult to determine precisely how both total and specific antibody concentrations relate to that in the individuals serum. These include the efficiency of absorption and elution of oral fluid from the collection device and the extent to which the antibody rich crevicular fluid is targeted by the collector when using the device (139). It is also likely that the extent to which antibody transudes into the oral cavity from blood capillaries beneath the gingival crevice will vary between individuals. Therefore antibody levels in oral fluid may be regarded only as a reflection of those in the subject's plasma. However, a degree of quantitation may be achieved if the specific antibody result is compared to the total antibody concentration of the oral fluid sample. Careful evaluation using an appropriate panel of paired sera and oral fluids will determine whether this approach is feasible.

The agreement between results of oral fluid samples and matching sera provided by the children from north Hertfordshire was sufficiently high for both rubella and parvovirus B19 specific IgG oral fluid tests to be useful for epidemiological purposes.

For the Ethiopian population, the agreement of rubella specific IgG results from oral fluid with those on matching sera was considerably lower. This suggests that the use of oral fluid for measuring rubella specific IgG lacks sensitivity for the Ethiopian study population. Nokes et al (1998) (128)observed a trend of decreasing rubella specific IgG in oral fluid with increasing age for this population. This suggests that if older age groups (>20 years) are to be screened for rubella specific IgG using oral fluid more sensitive virus specific antibody assays will be required. This issue is considered in more detail in Chapter 2 as the relatively small number of samples spanning the wide age range for the Ethiopian population makes a formal investigation of the effect of age on assay performance inappropriate here. The higher agreement for rubella specific IgG in oral fluids and matching sera found when evaluating the oral fluid samples provided by the children from north Hertfordshire is most likely the result of these participants having recently received MMR vaccine, these individuals therefore having a relatively high proportion of total antibody that is rubella specific.

There was little to discriminate between the three devices in terms of obtaining a qualitative rubella specific IgG result in both study populations. The results from the samples from the Ethiopian population are more likely to be reliable, however, as each of the 38 individuals provided an oral fluid collected using each of the three devices being assessed whilst the children from north Hertfordshire were randomised to use only one of the three devices.

A comparison of duplicate oral fluid samples

Duplicate oral fluid samples were collected from the rural Ethiopian population using the Oracol and OraSure devices. Comparisons of total IgG, IgM and rubella specific IgG antibody in duplicate oral fluids for each device was made using rank correlation. Results summarised in Table 9 show the correlation of total IgG concentration and rubella specific IgG for duplicate oral fluids collected using the Oracol was considerably higher than for oral fluids collected using the OraSure device. The correlation of total IgM was higher for duplicate samples collected by OraSure than for Oracol, though the confidence intervals were wide. These results therefore suggest that the Oracol device provided oral fluid samples of more consistent quality than the OraSure. This may reflect the way in which the Oracol device is used. It targets the gums and is used vigorously suggesting that it may be more likely to absorbe antibody rich crevicular fluid and so provide samples that contain more consistent levels of antibody. However, further investigations are required to examine consistency of sample due to the relatively small numbers of duplicate samples studied, particularly for the Oracol device (n = 13).

ACCEPTABILITY

All participants from both populations in this study found each make of oral fluid collection device simple to use. The Oracol device was preferable for use in the field as it was the easiest to administer and most acceptable to participants. Some participants from rural Ethiopia expressed a dislike of the taste of the OraSure device and some difficulty was experienced by younger participants in complying with the manufacturers instructions to position the Omni-SAL under the tongue for the

necessary time for the saturation indicator to turn blue. There was also a potential risk with young children chewing and biting off the Omni-SAL swab (128).

Further considerations which may be neccessary concern the laboratory processing needed to extract the oral fluid from each type of collection device. Whilst the procedures are relatively simple for all three devices, that for the Oracol is most time consuming and complex which may be a limiting factor that needs to be considered for basic laboratories in the developing world. There is also no preservative fluid provided with the Oracol device which may be a disadvantage when the used device cannot be quickly transfered from the field to the laboratory for processing.

CONCLUSIONS

- This study has confirmed that oral fluid may be used as a substitute for serum for sero-epidemiology and has high potential for determining the need for immunisation and the vaccine delivery strategy.
- There was little difference in the rubella specific IgG concentration in oral fluid for each type of collection device for qualitiative analysis.
- The Oracol device provided oral fluid samples with the highest concentrations of total IgG and IgM.
- The Oracol device provided the most consistent sample in terms of the total and specific immunoglobulins examined.
- The Oracol device was acceptable to the most participants.

Coupled to its lower cost compared to OraSure and Omni-SAL, these data suggest that the Oracol is the oral fluid collection device of choice for studies involving rural Ethiopia and young children in the UK.

APPENDIX 1

1.1 Measurement of total IgG and IgM concentration in oral fluid.

1.1a Preparation of IgG standard curve

- A calibration curve using doubling dilutions from 2.5 mg/L to 0.039 mg/L is prepared in PBST as described below. Each standard is tested in duplicate in each assay or each plate.
- Check the concentration of the standard in use. In a separate microtitre plate prepare a 2.5 mg/L standard. For example dilute a 45 mg/L calibrant 1 in 18 (10ul to 170ul PBST).
- Prepare the remaining standards by doubling dilutions, from the 2.5 mg/L diluted standard, in PBST (50ul standard + 50ul PBST). A new pipette tip must be used for the preparation of each dilution.

1.1b Assay procedure

- This assay is only accurate in the approximate range of 0.1 mg/L to 10 mg/L. Therefore, if a sample is likely to contain a concentration of IgG outside this range it must be tested at a different dilution. Further advice on appropriate sample dilutions is provided at the end of this section.
- A microtitre plate is coated with rabbit antibody to human IgG (gamma chain specific, Dako Ltd., Ely, United Kingdom) diluted 1:1000 in coating buffer (see 1.2a for coating buffer preparation) for 2 hours on a rotary shaker at room temperature. Leave overnight at 4^0C prior to use.
- Add 40ul of PBST to the wells. Add 10ul of oral fluid to its allocated microtitre well.

- After the addition of all the samples add a further 10ul of PBST to wells A1 and A2 to act as blanks for the assay. Add 10ul of each of the standards (in duplicate) to their allocated wells in columns 1 and 2.
- Cover the plate with a plate sealer and incubate at 37^{0}C for 2 hours.
- Wash the microtitre plate 4 times with a Wellwash (or similar), ensuring "top aspiration" is occurring.
- Prepare a 1 in 10,000 dilution of rabbit HRPO conjugated anti-human IgG (Dako) [1ul + 10ml, 10mls per plate], and add 100ul to each well.
- Cover the plate with a plate sealer and incubate at 37^{0}C for 30 minutes.
- Wash as descibed previously but include an additional wash, giving a total of 5 washes.
- Add 100ul of TMB substrate to each well, cover the plate with a plate sealer and incubate in the dark at room temperature for 25 minutes.
- Stop the reaction with 0.25M H_2SO_4 and read in a plate reader at 450nm (reference 620nm) using wells A1 and B1 as blanks. It is recommended that a reader capable of reading OD values >3.0 is employed in order to extend the dynamic range.
- Appropriate software (e.g. Mikrotek-Laborsysteme) should be used to analyse the data and to produce the standard curve against which oral fluid sample concentrations are determined.

Quantification of total IgG in oral fluid samples with concentrations >10mg/L: As the assay is capable of accurate quantification over only a relatively small linear region, if actual concentrations are required it is necessary to pre-dilute the samples which have given OD values above the linear range of the assay. Therefore prepare a dilution of the samples in PBST outside of the reaction tray, transfer them, and test as described

above. The actual dilution required will be dependant upon the actual concentration of the sample.

This method was adapted for measuring the total IgM concentration (mg/L) in oral fluid by using a test plate coated with an anti-human IgM antibody (Dako Ltd) at 1:1000, and diluting the oral fluid 1:10 in PBST. The human IgG calibrator was substituted with a human IgM calibrator (The Binding Site Limited, Birmingham, UK) and a calibration curve constructed with human IgM concentrations ranging from 2 mg/L to 0.0035 mg/L. An anti-human IgM peroxidase conjugate (Dako Ltd) was used at a dilution of 1:3000 and the TMB substrate added for 15 minutes before the colour reaction was stopped.

Limitation of the procedure: At present there is no feasible way of confirming the specificity of reactions. It has to be assumed that, as for any immunoassay, there may be some false reactions.

Useful notes:

- The critical part of this assay is the preparation of the calibration curves. Great care is needed when preparing the dilution series.
- When calculating the actual IgG and IgM concentration do not forget to compensate for the sample dilution.

1.2 IgG antibody capture radioimmunoassay (GACRIA) for rubella

1.2a Bead coating

- Preparation of coating buffer: Dissolve 0.8g of sodium carbonate Na_2CO_3 (15mM) and 1.47g of sodium hydrogen carbonate $NaHCO_3$ (35mM) in 500mls sterile distilled water. The pH should be 9.6.
- Take an appropriate number of polystyrene beads (Northumbria Biologicals P201).
- Coat beads with rabbit antibody to human IgG (gamma chain specific, Dako Ltd., Ely, United Kingdom) diluted 1:1000 in coating buffer for 2 hours on a rotary shaker at room temperature. Leave overnight at 4^0C prior to use.

1.2b Preparation of calibration curve

- A serum sample, strongly positive for rubella IgG, is used as an arbitary 100u control.
- Further dilutions of this 100u control in human serum negative for rubella specific IgG (NHS) are made to give 33u, 10u, 3.3u, 1.0u and 0.33u controls.

1.2c Testing serum and oral fluid samples

- For each assay run 4 wells of NHS and a calibration curve are used. Where serum and oral fluid samples are tested in the same assay duplicate control plates are required.
- Specimens: serum samples are diluted 1:100. The diluent used is phosphate buffered saline (PBS) containing 10% foetal calf serum (FCS) and 0.2% Tween 20 (T20). Oral fluid samples are tested neat.
- Coated beads are washed 3 times in PBST (T20 0.05%) and added to sample and control wells.
- Incubate at 37^0C for 3 hours.

- Wash x1 using Qwikwash (Abbott Laboratories) in PBST.
- Add 200ul rubella haemagglutinin antigen (Judith Strain) prepared by DMR, CPHL, diluted 1:15 in PBS containing 10% FCS and 1% T20.
- Incubate overnight at 4^0C.
- After washing beads as described previously, add 200ul anti-rubella monoclonal antibody to each sample diluted 1:50,000 in PBS containing 10% FCS, 2% NHS, 5% normal rabbit serum (NRS) and 0.1% T20.
- Incubate for 2 hours at 37^0C.
- After washing beads as described previously, add I^{125} labelled anti-mouse IgG (Amersham code no. IM131) diluted to give approximately 80,000 - 100,000 counts per minute in 200ul. Dilute in PBS containing 10% FCS, 2% NHS, 5% NRS, 5% normal goat serum (NGS) and 0.2% T20.
- Incubate for 2 hours at 37^0C.
- After washing as before, transfer the beads to counting tubes and measure the bound I^{125} for 2 minutes in a gamma counter.
- Test results are calculated as the total bound radioactivity of each specimen divided by the mean radioactivity bound by the negative serum controls and expressed as test:negative (T/N) ratios. A cut off T/N of 2.1 was used. Results can also be expressed in units by reference to the calibration curve.

1.3 Behring Enzygnost anti-rubella virus IgG ELISA: enzyme immunoassay for the detection and quantitative determination of human IgG antibodies to rubella virus in serum and plasma.

1.3a Preparation of the reagents

- Bring all the reagents and test samples to 18 - 25^{0}C before starting.
- For each test plate dilute 20ml Washing Solution POD to 400ml with distilled water.
- Pre dilute the reference(s) and test samples 1+20 with Sample Buffer POD and mix well.
- Working Conjugate Solution: dilute the Anti-human IgG/POD Conjugate with Conjugate Buffer Microbiol (1+50), shaking gently to mix.
- Working Chromogen Solution: for each test plate, dilute 1ml Chromogen TMB with 10ml Buffer/Substrate TMB in the empty bottle supplied with the kit and store protected from light.

1.3b Test Procedure

- Assay scheme: ascertain the required number of wells (= No. test samples + No. determinations of the reference P/N). Reference P/N is run as the first and last sample in the assay series, or as the first and last sample in each test plate in a large assay series.
- Introduce buffer: pipette receiving volumes of 200ul Sample Buffer POD into each well needed in the test plate(s).
- Dispense samples: pipette 20ul/well of the prediluted Reference P/N into the first pair of antigen (Ag)/control antigen (CoAg) wells (A/1 and A/2). Afterwards pipette 20ul/well of prediluted sample into the next pair of Ag/CoAg wells, continuing in the same manner for each sample. At the end of the series or test

plate again pipette 20ul/well of the prediluted Reference P/N into a pair of Ag/CoAg wells. Seal the finished test plate.

- Incubate for 1 hour at 37°C.
- Wash: aspirate all wells; introduce approx 0.3ml of diluted Washing Solution POD into each well, aspirate and repeat the cycle three more times.
- Dispense conjugate: into each well pipette 100ul of the Working Conjugate Solution, seal plate.
- Incubate for 1 hour at 37°C.
- Wash as previously described.
- Dispense substrate: add 100ul of Working Chromagen Solution to each well and seal plate.
- Incubate for 30 minutes at 18 - 25°C protected from light.
- Stop reaction: add 100ul of Stopping Solution POD to each well.
- Photometric evaluation: the measurement wavelength is 450nm, and the recommended correction wavelength is 650nm. For each test sample and reference sample calculate the difference in absorbance, ΔA ($=A_{antigen} - A_{control\ antigen}$), obtained with the sample diluted as prescribed.

1.3c Internal Quality control

- The A values for each pair of Reference P/N well must be within the range defined by the lower and upper margins given in the table of values enclosed with the kit.
- The A values of the Reference P/N at the start and the end of the series must not deviate from the mean of these values by more than $\pm$ 20%.

1.3d Results (Quantitative Evaluation)

- Measurement correction: calculate the mean A value of the Reference P/N. Divide the nominal value of the Reference P/N (given in the Table of Values) by the mean A value. This gives the correction factor.
- Use the correction factor to multiply the absorbances of the samples in the series for which the correction factor was calculated. If several test plates are assayed, the correction factor has to be determined for each plate separately and used to correct the values for the corresponding plate.
- Calculation of the result: calculate the antibody activities by using the corrected ΔA readings of the following formula (α-method): **log10 IU/ml =** $\alpha . \Delta A^{\beta}$
- The values for the lot-dependant constants alpha (α) and beta (β) are given in the enclosed table of values.
- The antibody activity is stated in "IU/ml" based on the International Standard for Anti-Rubella Serum (2nd International Standard Preparation) of WHO.
- The limit of detection of the test is 4 IU/ml, equivalent to a signal of 0.1 A.

1.4 IgG antibody capture radioimmunoassay (GACRIA) for parvovirus B19

1.4a Bead coating (as described for rubella GACRIA using Dako anti-human IgG diluted 1/2000)

1.4b Preparation of calibration curve (as described for rubella GACRIA using a serum strongly positive for parvovirus B19 IgG as an arbitary 100u control)

1.4c Test Procedure

- For each assay run 4 wells of NHS and a calibration curve are used. Where serum and oral fluid samples are tested in the same assay duplicate control plates are required.

- Specimens: serum samples are diluted 1:100. The diluent used is phosphate buffered saline (PBS) containing 10% foetal calf serum (FCS) and 0.2% Tween 20 (T20). Oral fluid samples are tested neat.
- Coated beads are washed 3 times in PBST (T20 0.05%) and added to sample and control wells.
- Incubate at 37^{0}C for 2 hours.
- Wash x1 using Qwikwash (Abbott Laboratories) in PBST.
- Add 200ul baculovirus expressed B19 VP1 and VP2 capsids (provided by Dr S. Kajigaya, NIH Bethesda) diluted 1:10,000 in PBS containing 10% FCS and 1% T20.
- Incubate overnight at 4^{0}C.
- After washing beads as described previously, add 200ul anti-VP1/VP2 monoclonal antibody to each sample diluted 1:100,000 in PBS containing 10% FCS, 2% NHS, 5% normal rabbit serum (NRS) and 0.1% T20.
- Incubate for 2 hours at 37^{0}C.
- After washing beads as described previously, add I^{125} labelled anti-mouse IgG (Amersham code no. IM131) diluted to give approximately 80,000 - 100,000 counts per minute in 200ul. Dilute in PBS containing 10% FCS, 2% NHS, 5% NRS, 5% normal goat serum (NGS) and 0.2% T20.
- Incubate for 2 hours at 37^{0}C.
- After washing as before, transfer the beads to counting tubes and measure the bound I^{125} for 2 minutes in a gamma counter.
- Test results are calculated as the total bound radioactivity of each specimen divided by the mean radioactivity bound by the negative serum controls and expressed as test:negative (T/N) ratios. A cut-off T/N of 4.0 was used.

1.5 Dako IDEIA™ parvovirus B19 IgG ELISA: enzyme immunoassay for the detection and qualitative determination of human IgG antibodies to human parvovirus B19 in serum samples.

1.5a Preparation: determine the number of microwell strips needed. Given duplicate tests, between 12 and 45 specimens can be tested per kit, dependant on the number of specimens per run. One strip allows assaying of 1 patient specimen, with duplicate tests plus controls. Each additional strip allows duplicate tests of 4 specimens. All steps are performed at room temperature (20 - 25^{0}C).

1.5b Incubation of samples

- Place the required number of microwell strips in the plastic frame
- Dilute patient sera 1 + 100 by adding 10uL of serum to 1 mL of working strength Sample Diluent and Washing Buffer in a test tube
- First microwell strip: add 50uL of working strength Sample Diluent and Washing Buffer (vial No. 2) to wells A1 and B1; Cut-Off IgG Control (vial No. 3) to wells C1, D1 and E1; Positive IgG Control (vial No. 4) to well F1; and 50 uL of diluted patient serum to G1 and H1.
- Additional microwell strips: add patient specimens only. For each patient, pipette 50 uL of diluted serum into each of two adjacent wells. If more than one strip is used in an assay, the volumes must be multiplied by the number of strips.
- Add 300 uL of working strength Sample Diluent and Washing Buffer to a test tube.
- Mix the contents of vial No. 5 (biotinylated B19 Antigen, previously reconstituted) and add 200 uL to the test tube. Mix.
- Add 50 uL of diluted Biotinylated B19 Antigen to each well. Return vial No. 5 to the refrigerator immediately after use.

Cover the microwell strips with a plastic lid and incubate for 30 minutes at room temperature (20 - 25°C) on an orbital shaker set at 500 rpm.

1.5c Washing

- Wash the wells with 4 times with working strength Sample Diluent and Washing Buffer using an ELISA washer. Good washing with complete filling and emptying of the wells is essential for optimal results.

1.5d Incubation with Conjugate, Anti-IgG

- Add 100 uL of Conjugate, Anti-IgG (vial No. 6) to each well.
- Cover the microwell strips with a plastic lid and incubate for 1 hour at room temperature (20 - 25°C) on an orbital shaker set at 500 rpm.

1.5e Washing

- Wash the wells as described previously

1.5f Incubation with Substrate A and B, reading of results: it is recommended to use an 8-channel pipette throughout the following steps. Use separate reagent reservoirs for substrate A and Substrate B with only the requisite amount of reagent.

- Add 50 uL of Substrate A (vial No. 7) to each well.
- Add 50 uL of Substrate B (vial No. 8) to each well.
- Incubate the microwell strips for 10 minutes at room temperature (20 - 25°C), covered with a plastic lid and protected from the light.
- Stop the reaction by adding 100 uL of Stop Solution (vial No. 9) to each well.
- Read the absorbance (OD) of each microwell at 450 nm. Blank on air. For dual wavelength readers use a reference filter at 630 - 650 nm. Read the absorbance within 60 minutes after the addition of Stop Solution.

1.5g Calculation of results

- Buffer control: calculate the mean absorbance value (OD value) of the two buffer control wells.
- Cut-off Control: calculate the mean OD value of the three Cut-off Control wells. The OD value of the individual wells should not differ more than 15% from the mean. If one of the three OD values differs by more than 15% from the mean, it should be omitted and the mean value re-calculated.
- Patient specimens: calculate the mean OD value for each patient specimen. The OD values should not differ more than 15% from the mean. However, a difference of more than 15% may be accepted if both test wells show a negative result (low OD values are measured with less precision).
- Quality control: check that the mean OD value of the buffer control is less than 0.100 but greater than 0.000. If the value is above 0.100, inadequate washing or contamination of Substrate A or B may be the cause. If the value is less than 0.000, the ELISA reader should be re-blanked on air, and the well re-read. The difference between the OD value of the Positive Control and the Cut-Off Control should be at least 0.800. If the difference is less than 0.800, inadequate washing, contamination of conjugate with serum, or incubation at too low temperature, in particular during substrate incubation, may be the cause. The shaker must also comply with the specifications stated in the working procedure in order to assure the test quality. If the quality control requirements are not satisfied the test results are invalid and the assay should be repeated.

*1.5h **Interpretation of results:*** Compare the OD values of specimens directlt with the OD value of the Cut-off Control. Interpret as follows:

- Negative for IgG antibodies to parvovirus B19: Specimen OD $\leq$ OD of Cut-off Control x 0.8

- Equivocal for IgG antibodies to parvovirus B19: Specimen OD is > than OD Cut-off Control x 0.8, but < the Cut-off Control x 1.2
- Positive for IgG antibodies to parvovirus B19: Specimen OD is ≥ OD of the Cut-off Control x 1.2

CHAPTER 2

THE DEVELOPMENT OF SIMPLE ELISA ASSAYS FOR USE WITH ORAL FLUID

Objectives:

- To develop simple, sensitive and robust ELISA-based oral fluid antibody detection methods for rubella and anti-HBc IgG which can be used to replace existing radioimmunoassays and will be suitable for use in both developing and developed countries.
- The rubella ELISA developed will be suitable to replace the radioimmunoassay currently used to detect rubella specific IgG antibody in oral fluid samples within the MMR surveillance programme in England and Wales.
- To assess, evaluate and compare against well established commercial serum ELISA the performance characteristics of ELISA-based oral fluid antibody assays developed in this study.

MATERIALS AND METHODS

Rubella GACELISA

Oral fluid collection.

Oral fluid was collected and extracted as previously described, using the Oracol, OraSure and Omnisal devices.

Sera, oral fluid and paired serum/oral fluid panels.

All samples were stored at -20^{0}C until required for testing.

Control Sera.

RV IgG positive (256 IU/ml) and negative sera (< 4 IU/ml) from healthy blood donors were identified using a commercial ELISA kit (Behring Enzygnost, Behringwerke AG, Marburg, Germany), as previously described. The WHO 80 IU/ml 2nd international RV antibody standard (National Institute of Biological Standards and Controls [NIBSC], Potters Bar, UK) was used to assess assay sensitivity.

Serum/oral fluid pairs.

Assay development and evaluation

Four panels comprising of 197 serum/oral fluid pairs were used (Table 17). All sera were tested using the Behring ELISA as described above.

- **Panel 1** consisted of 97 pairs positive for serum RV IgG antibody from children (aged 3.5 - 5 years) who had recently received MMR vaccine as part of a pre-school boost (PSB) study previously described in chapter 1. In total 36 of the oral fluids were collected using Oracols; 30 using the Orasure device (Epitope Inc, Beaverton, UK); 26 using the Omni-SAL™ device (Oral fluid Diagnostics Systems

Ltd, Singapore) and for five oral fluids the collection device used was not recorded. The oral fluids were also tested by RV IgG capture radioimmunoassay (GACRIA) as previously described.

- **Panel 2** consisted of 24 pairs negative for serum RV IgG antibody from a study of congenital rubella in southern India (51), the oral fluids having been collected using the Orasure device and tested by RV GACRIA.
- **Panel 3** consisted of 76 pairs, 14 negative and 62 positive for serum RV IgG antibody, from the Christian Medical College, Vellore, India. The oral fluids were collected using the Omni-SAL™ device and tested by RV GACRIA. This panel was categorized by age and subdivided into two further panels:

  panel 3a 55 serum/oral fluid pairs from subjects aged 17-34 years

  panel 3b 21 serum/oral fluid pairs from subjects aged 5 months to 10 years.

Assay use in a field study

A panel of 831 paired serum/oral fluid samples were obtained from persons aged between 0 and 84 years from the rural district of Butajira, Ethiopia. Oral fluid was collected and extracted as previously described, using the Oracol device. All sera were tested using the Behring ELISA as described above and oral fluid samples were screened for anti-RV IgG by FITC/anti-FITC GACELISA.

Anti rubella-FITC conjugate.

Monoclonal antibody (MAB) to RV haemagglutinin (177) (Laboratory of Microbiological Reagents (LMR), Central Public Health Laboratory) was purified by a modification of the caprylic acid precipitation method as described by Steinbuck and Audran (174) and Samuel et al (165). Briefly, ascitic fluid was clarified by

centrifugation and filtration and the pH adjusted to 4.8 using 0.1 M acetic acid. Whilst continuously stirring at room temperature, 77ul of caprylic acid per ml of ascitic fluid was added and the resulting precipitate removed by centrifugation. The supernatant was collected and the pH adjusted to neutral with TRIS buffer. An equal volume of saturated ammonium sulphate was then added and the solution left to stand overnight at 4^0C. The resulting precipitate was recovered by centrifugation and redissolved in a 0.1 M carbonate-bicarbonate buffer (pH 9.25) containing 0.15 M NaCl. 1ml of the purified monoclonal antibody was dialysed against 100ml of the 0.1M carbonate-bicarbonate buffer.

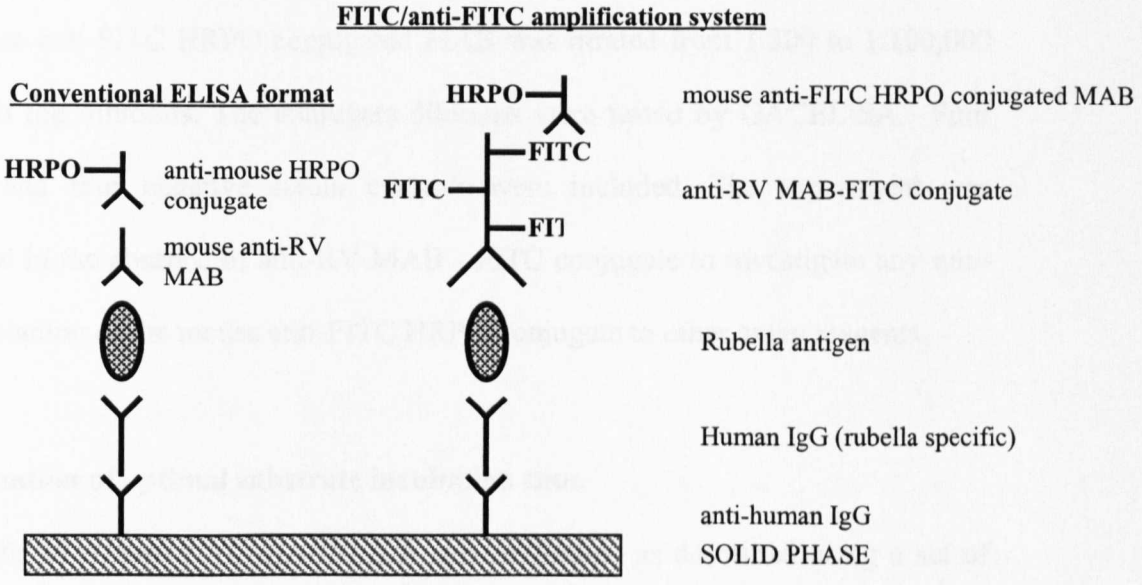
Conjugation with fluorescein isothiocyanate (FITC) was carried out as described by Samuel et al (165). Briefly, 1 mg of purified monoclonal antibody was added to 0.5 ml aliquots of continuously stirred 0.1 M carbonate-bicarbonate buffer, pH 9.25, containing 0.15 M NaCl. To these a 5 mg/ml solution of FITC in absolute ethanol was then added over a 5 minute period and the conjugation reaction allowed to proceed for 45 minutes at room temperature whilst being continuously stirred. The FITC conjugated monoclonal antibody was separated from free FITC by gel filtration on a PD 10 column (Pharmacia) and stored at 4^0C.

Rubella FITC/anti-FITC GACELISA.

Development and Optimisation

The assay format is shown in Fig. 6.

Fig 6: Conventional ELISA and the FITC/anti-FITC amplification system



A series of experiments to optimise assay performance were performed:

Varying RV haemagglutinin concentration

RV haemagglutinin was titrated from 1:5 to 1:150 using duplicate positive and negative serum controls for each concentration of RV haemagglutinin examined.

Determination of optimal anti-RV MAB - FITC conjugate concentration

The anti-RV MAB - FITC conjugate was titrated from 1:300 to 1:100,000 using half log dilutions. The conjugate dilutions were tested by GACELISA. Four positive and

four negative serum controls were included and the experiment performed in both the presence and absence of antigen.

Determination of optimal mouse anti-FITC HRPO conjugated MAB concentration

The mouse anti-FITC HRPO conjugated MAB was titrated from 1:300 to 1:100,000 using half log dilutions. The conjugate dilutions were tested by GACELISA. Four positive and four negative serum controls were included. The experiment was performed in the absence of anti-RV MAB - FITC conjugate to investigate any non-specific binding of the mouse anti-FITC HRPO conjugate to other assay reagents.

Determination of optimal substrate incubation time

The rubella FITC/anti-FITC GACELISA was performed as described using a set of six duplicate positive and negative serum controls. TMB substrate was added to each of the six sets of control sera and the colour reaction then stopped at five minute intervals, from 5 minutes to 30 minutes, by the addition of 2M H_2SO_4 to one of the sets of serum controls. The optical density was read immediately after the colour reaction had been stopped.

Evaluation of GACELISA performance with samples incubated on a plate shaker

The rubella FITC/anti-FITC GACELISA was performed as described using duplicate titrations of the WHO 80 IU/ml international standard diluted in NHS from 80 IU/ml to 0.625 IU/ml. One dilution series was incubated shaking at 500 rpm on a plate shaker and the other being incubated stationary. Also included as a comparison was a

system using only the mouse anti-RV MAB and an anti-mouse HRPO conjugate (DAKO) rather than the FITC/anti-FITC system.

Final Procedure

- **Wells of microtitre plates (Immuno Module Maxisorb U8 immunoplates, Life Technologies Ltd, Paisley, UK) were coated with 100ul of rabbit antibody to human IgG (gamma chain specific, Dako Ltd, U.K) diluted 1/1000 in 0.05 M sodium carbonate/bicarbonate buffer pH 9.6. and incubated at 37^{0}C for 18 h.**
- **After washing with phosphate buffered saline (PBS) containing 0.05% Tween 20 (T20) the wells of each plate were incubated at 37^{0}C on a plate shaker at 500 rpm successively with 100ul of the following:**

1. undiluted oral fluid or a 1 in 100 dilution of serum in PBS containing 10% fetal calf serum (FCS) and 0.2% T20 for 30 mins;
2. RV haemagglutinin (LMR) diluted 1 in 10 in PBS containing 10% FCS and 0.2% T20 for 1 h;
3. a 1/500 dilution of the anti-RV MAB - FITC conjugate in PBS containing 10% FCS, 5% normal rabbit serum (NRS), 2% human serum negative for anti-rubella IgG (NHS) and 0.2% T20 for 2 h;
4. mouse anti-FITC horse raddish peroxidase (HRPO) conjugated MAB (Chemicon International Inc. Temecula, USA) diluted 1/18,000 in PBS containing 10% FCS, 10% NRS, 2% NHS and 1% T20 for 25 mins.

- **Finally, 100ul of a 42mM 2`2` 4`4` tetramethyl benzidine (TMB) substrate solution in a 0.1M citrate/acetate buffer (pH 6.0) with 0.075% 20 volumes H_2O_2 (6% w/v) was added to each well and the plate left to stand at room temperature in the dark for 20 mins.**

- **The reaction was stopped by the addition of 50ul 2M H_2SO_4 to each well and the optical density measured immediately at 450 nm (620 nm reference) [$OD_{450/620}$] using a Labsystems iEMS plate reader.**
- **Between each stage of the assay the wells were washed 5 times with PBS T20 (0.05% T20) using a Denley wellwash 4 Mk2.**
- **Included as controls were 8 wells each of the serum strongly positive for RV IgG, NHS and the WHO 80 IU/ml international standard diluted in NHS to give 15 IU/ml.**

Determination of cut-off value.

The setting of an appropriate cut-off value is a particularly important aspect in the development of an assay as the assay performance will be judged by this. One factor that influences the choice of an assay cut-off is the purpose for which the assay under development is designed to be used. Assays primarily developed to be used for diagnosis in the individual will often employ a relatively high cut-off that errs on the side of higher specificity at the expense of sensitivity to avoid wrongly categorising true negatives as weakly positive. A good example of this are assays used for the detection of RV specific IgG in determining whether or not an individual has protective levels of antibody. Since it is critical that susceptible women of children bearing age are accurately identified and offered vaccination to avoid the possibility of a congenital infection during pregnancy, a high cut-off is used even though this may result in classifying certain low positive results – that may actually reflect the true immune status of an individual - as negative. In contrast screening assays designed to estimate prevalence within a population can afford to use a lower cut-off

to obtain best agreement with a gold standard assay and provide the highest sensitivity and specificity.

A further factor to consider when setting an appropriate assay cut-off value is the availability of well-characterised positive, and particularly negative, samples assessed using a gold standard assay. The mean result of a population of negative samples confirmed as such by a gold standard assay plus either two or three standard deviations is a commonly used method to set a cut-off value (159). However, it should be stressed that the estimate of standard deviation is subject to error as well, and depends on the number of responses from which it was calculated (88).

The radioimmunoassay used to measure rubella specific IgG in oral fluid (144) expresses results as a test sample to negative control ratio (T:N). Since signals are generated as measured counts of radioactivity over a specific period, the assay results typically range from a T:N of <1.0 for negative samples to a T:N that is typically >100 for a sample strongly positive for rubella specific IgG, samples being considered as positive with a T:N >2.1. The spectrum across which results fall, from negative to strong positive, represents the "dynamic range" of an assay. Signals generated by ELISA assays are measured as optical density values and if results are expressed as T:N values, they typically range from <1.0 for negative samples to ~20.0 for a strong positive. Therefore if results from ELISA assays are expressed as T:N ratios the dynamic range is small in contrast to radioimmunoassay. The advantage in having an assay with a large dynamic range is that it makes it easier to distinguish positive from negative results, particularly those that fall close to the assay cut off.

To enhance the dynamic range the results of the GACELISA were expressed as a corrected percentage (%P) of the $OD_{450/620}$ of the positive control serum included in each assay using the following formula:

Corrected Percentage (%P) = {[($OD_{450/620}$ of the sample - $OD_{450/620}$ of the negative control)/ ($OD_{450/620}$ of the positive control - $OD_{450/620}$ of the negative control)]} x 100

By incorporating the $OD_{450/620}$ of both the positive and negative controls, the formula allowed for interassay variation. The %P values may therefore range from <0% for those samples whose $OD_{450/620}$ is less than that for the negative control to >100% for those samples whose $OD_{450/620}$ is greater than that of the positive control.

A cut-off was estimated for the GACELISA using two standard deviations of the result from oral fluids from 26 subjects negative for serum RV IgG antibody by Behring Enzygnost ELISA plus the mean result for all 26 samples. This gave a cut-off value of 2.7%: samples with $\%P \geq 2.7$ were considered RV IgG positive; those with $\%P < 2.7$ were considered RV IgG negative.

This method generated a working cut-off. For assays designed to be used for determining immunity in the population a more appropriate and accurate cut-off may be set by using mathematical and statistical techniques such as mixture modelling (137) and receiver operating characteristic (ROC) curves (23). A disadvantage of these techniques is that they require the assay to have been already used extensively on a suitably large population of samples. The appropriateness and suitability of the cut-off value chosen will be appraised later using ROC curves (p. 106, p. 142).

Statistical Methods.

Assay performance was evaluated by four methods:

1. Kappa statistic : This evaluates the degree of agreement between two measurements and is used when neither assay is universally accepted as a gold standard. A kappa statistic of 1 indicates perfect agreement, one of 0 corresponds to no agreement and one of -1 indicates perfect negative agreement. 95% confidence intervals around kappa were also calculated. If this interval is above 0 it can be concluded that the methods show more agreement than expected by chance. Kappa statistics were calculated using the fomula:

$$K = (P_{obs} - P_{exp})/(1 - P_{exp})$$

where P_{obs} is the observed proportion of agreement between the two methods and P_{exp} the proportion of agreement expected by chance (55).

**2. Spearman`s Rank Correlation** : This is a non-parametric measure of the degree of association between two variables. The values of each variable are independently ranked and the measure based on the differences between the pairs of ranks of the two variables. Spearman`s rank correlation was calculated using the formula :

$$r = 1 - [6\sum d^2/(n(n^2-1))]$$

where **d** is the difference between each pair of ranks and **n** the number of subjects. A value of 1 corresponds to perfect agreement between the ranks of the two variables, 0

corresponds to no relationship, and -1 corresponds to a perfect inverse agreement between the ranks. 95% confidence intervals around r_s were also calculated (5).

3. Exact binomial test : The binomial distribution was used to calculate the p-value for comparing the agreement of results of the two oral fluid assays with the matching serum results (5).

4. Paired t-test : A comparison of assay results when incubating the microtitre plate shaking at 37°C and stationary at 37°C was made using a paired t-test (5).

Sensitivity, specificity, positive and negative predictive values (91).

The following fourfold table (Table 15) is useful in defining and calculating sensitivity, specificity, positive and negative predictive values for antibody assays.

Table 15. Sensitivity, specificity, positive and negative predictive values

Screening test results	True status		TOTAL
	Positive	Negative	
Positive	*a*	*b*	*a+b*
Negative	*c*	*d*	*c+d*
TOTAL	*a+c*	*b+d*	*a+b+c+d*

a: True positives detected by the screening test

b: False positives detected by the screening test

c: False negatives detected by the screening test

d: True negatives detected by the screening test

Sensitivity: This is a measure of the probability that any given case will be identified by a screening test and is defined as the proportion of true positives in the screened population who are identified as so by the screening assay.

[Sensitivity = *a/(a+c)*]

Specificity: This is a measure of the probability that a true negative will be correctly identified by the screening test and is defined as the proportion of true negatives in the screened population who are identified as so by the screening assay.

[Specificity = *d/(b+d)*]

Positive (PPV) and negative (NPV) predictive values: These are defined as the proportion of cases of a condition accurately identified by a screening test.

[PPV = *a/(a+b)*]

[NPV = *d/(c+d)*]

When calculating sensitivity, specificity, PPV and NPV values it is assumed that the serum assay provided the definitive or "true to nature" result. It must be recognised, however, that this may not necessarily be the case. Sensitivity and specificity are absolute values associated with the screening test whereas PPV and NPV are variable, dependent upon the prevalence of "true" positive or negatives in the population the assay is used to screen.

Receiver Operating Characteristic (ROC) Curves

Receiver operating characteristic (ROC) curves are a plot of the true positive proportion (sensitivity) of a population against the false positive proportion (specificity) as detected by the screening assay when evaluated compared to a "true" or "gold standard" result. In this instance "true" results were generated by commercial ELISA assays (Behring Enzygnost for RV specific IgG and Hepanostika for anti-HBc) on matching serum samples to the oral fluid samples used. ROC analysis therefore provides a graphical representation of the balance between sensitivity and specificity of an assay. Ideally, both sensitivity and specificity would be 100% but this is rarely - if ever - achieved. ROC curves can therefore be used to assist with setting an assay cut-off though it is also important to consider the intended use of the assay. Since the assays developed in this study are screening assays for determining levels of immunity in the population a cut-off which gives maximum sensitivity and specificity (i.e a cut-off in which sensitivity is maximised to a level of minimum effect on specificity) should be chosen (23).

ROC curves are constructed by evaluating the sensitivity and specificity of an assay across a range of cut-off values with respect to a true or gold standard result. Sensitivity is then plotted against 1-specificity. A perfect test (100% sensitivity and 100% specificity) will generate a curve that passes through the ideal point (0, 100) in the upper left corner of the graph. The best cut-off for a screening assay (in which sensitivity is maximised to a level of minimum effect on specificity) will therefore be illustrated by the point on the curve which lies closest to the ideal point. Since the curve will have a gradient of 1.0 at this point it can be found by drawing a line of 45^0 at a tangent to the curve.

Anti-HBc GACELISA

Sera, oral fluid and paired serum/oral fluid panels.

All samples were stored at -20^{0}C until required for testing.

Oral fluid samples

Four oral fluids were provided by Virus Reference Division (VRD) staff. Two were from persons confirmed as anti-HBc IgG positive and were collected by dribbling into a sterile plastic container, and two were from persons with no history of hepatitis and were collected using Oracols as previously described.

Oral fluids (n = 334) were obtained from the Anonymous HIV Laboratory, VRD, and had been collected using the Salivette device (Sarstedt, Leicester, UK) as described by Mortimer and Parry (119). Briefly, the Salivette consists of a cylindrical swab of cotton wool contained in a plastic double tube. The subject must chew on the swab so that oral fluid surrounding the gums is absorbed, which is then placed into the internal tube. This is then centrifuged so the oral fluid can be recovered from the bottom of the outer tube.

Serum Samples.

The Hepatitis and Retrovirus Laboratory (HRL), VRD, provided 23 sera which had previously been screened for anti-HBc antibody by a commercial ELISA kit, the Hepanostika anti-HBc Uni-Form Microelisa system (ORGANON Teknika) according to the manufacturers instructions. This assay works on a competitive principle (see Fig. 24, p. 158). Briefly the test sera and controls were added undiluted to the test plate together with the conjugate provided. After incubation for the appropriate time,

the provided substrate solution was added to each test well. The colour reaction was stopped by the addition of provided stop solution and the absorbance read at 492 nm. The method is described in full in Appendix 2.1 (***pp. 176-178***).

Paired serum/oral fluid samples

Of the panel of 820 paired serum/oral fluid samples obtained from persons aged between 0 and 84 years from the rural district of Butajira, Ethiopia, 530 had sufficient volume remaining for screening for anti-HBc IgG. All sera were tested by the Hepanostika anti-HBc Uniform Microelisa system (Organon Teknika) as described above, and oral fluid samples were screened for anti-HBc IgG antibody by GACELISA.

Measurement of total anti-HBc antibody in oral fluid

The total anti-HBc concentration in oral fluid samples was measured using an adaption of the ICE HBc Detection Pack (Murex Diagnostics Limited, UK), and is descibed in full in Appendix 2.2 (***pp. 178-180***). Briefly, undiluted oral fluid samples and appropriately diluted controls were added to the test plate. The appropriate dilution of supplied conjugate was then added followed by substrate solution. The colour reaction was stopped by adding 0.5 M H_2SO_4 and the absorbance read 15 minutes later at 450nm.

Development and Optimisation

A series of experiments to optimise assay performance were performed.

Titration of serum samples

- The anti-HBc GACELISA was performed as described titrating a serum sample strongly positive for anti-HBc antibody and a serum sample negative for anti-HBc (determined using the Hepanostika anti-HBc Uni-Form microelisa system) from 1:100 to 1:100,00 using half log diluitions in NHS.

Titration of anti-mouse HRPO conjugate

- The anti-mouse HRPO conjugate was titrated sequentially from a 1:1000 dilution to one of 1:10,000. This experiment was then repeated examining a range of anti-mouse HRPO conjugate dilutions from 1:3500 to 1:5000. Anti-HBc positive and negative serum controls at a dilution of 1:100 were included.

Simultaneous titration of rHBcAg and serum

- The rHBcAg was titrated from 1:1000 to 1:3,000,000 and a serum strongly positive for anti-HBc from 1:100 to 1:300,000, both using half log dilutions.

Titration of mouse anti-HBc MAB

- The anti-HBc GACELISA was performed as described using positive and negative control sera (identified using the Hepanostika anti-HBc Uniform Microelisa system) and the mouse anti-HBc MAB titrated from 1:1000 to 1:3,000,000 using half log dilutions.

Simultaneous titration of mouse anti-HBc MAB and serum

- The mouse anti-HBc MAB was titrated from 1:10,000 to 1:300,000 (using intervals of 50,000) and a serum strongly positive for anti-HBc titrated from 1:100 to 1:300,000 using half log dilutions. A negative control serum, used at a dilution of 1:100 was also included for all dilution factors of mouse anti-HBc MAB.

Titration of oral fluid samples

- The anti-HBc GACELISA was performed as described using four oral fluid samples, two from subjects positive for anti-HBc and two from subjects anti-HBc negative. The oral fluids were titrated from neat to a 1:32 dilution, using doubling dilutions in NHS. Also included were two serum controls, one positive for anti-HBc and the other negative, used at a 1:100 dilution.

Investigating sources of non-specific binding

- The anti-HBc GACELISA was carried out as described leaving out various reagents at each stage so all permutations were covered to identify which reagent was binding to which non-specifically. The experiment was then repeated including 2% human serum negative for anti-HBc IgG (NHS) in the anti-HBc monoclonal antibody diluent.

Final procedure

- **Wells of microtitre plates (Immuno Module Maxisorb U8 immunoplates, Life Technologies Ltd, Paisley, UK) were coated with 100ul of rabbit antibody to human IgG (gamma chain specific, Dako Ltd, U.K) diluted 1/1000 in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6, and incubated at 37^{0}C for 18 h.**
- **After washing with phosphate buffered saline (PBS) containing 0.05% Tween 20 (T20) the wells of each plate were incubated at 37^{0}C on a plate shaker at 500 rpm successively with 100ul of the following:**

1. undiluted **oral fluid** or a 1 in 100 dilution of **serum** in PBS containing 10% fetal calf serum (FCS) and 0.2% T20 for 30 mins;
2. **recombinant HBc antigen** (rHBcAg) (Murex) diluted 1 in 3000 in PBS containing 10% FCS and 0.2% T20 for 1 h;

3. a 1/100,000 dilution of a mouse **anti-HBc MAB** (Murex) in PBS containing 10% FCS, 2% human serum negative for anti-HBc IgG (NHS) and 0.2% T20 for 2 h;
4. **anti-mouse horse raddish peroxidase (HRPO) conjugate** (Dako Ltd, U.K) diluted 1/3500 in PBS containing 10% FCS, 5% normal rabbit serum (NRS) and 0.2% T20 for 30 mins.

- **Finally, 100ul of a TMB substrate solution (Chemicon International Inc. Temecula, USA) was added to each well and the plate left to stand at room temperature in the dark for 30 mins.**
- **The reaction was stopped by the addition of 100ul 0.5M HCl to each well and the optical density measured immediately at 450 nm (620 nm reference) [$OD_{450/620}$] using a Labsystems iEMS plate reader.**
- **Between each stage of the assay the wells were washed 5 times with PBS T20 (0.05% T20) using a Denley wellwash 4 Mk2.**
- **Included as controls were 4 wells each of serum strongly positive for anti-HBc IgG and NHS.**

Determination of cut-off value.

Unlike the situation when determining a cut-off for the RV GACELISA, a panel of paired serum/oral fluid samples was not available where the serum result was confirmed as negative by a gold standard assay. Therefore an attempt to set a working cut-off value was made using the results of samples once a study population of paired serum/oral fluid samples had been screened. Of the 530 paired serum/oral fluid samples obtained from Butajira, Ethiopia, 183 sera tested negative by the Hepanostika anti-HBc Uni-Form Microelisa system. The mean $OD_{450/620}$ for the corresponding 183 oral fluids samples tested by GACELISA, plus 3 standard deviations, was 0.150

which was used as a working cut-off value (159). Three standard deviations were used rather than two, as was used for the RV GACELISA, due to the large number of paired serum/oral fluid sample available where serum tested negative by Hepanostika suggesting that the estimation of the standard deviation should be accurate.

Test sample $OD_{450/620}$ values were divided by the cut-off of 0.150, and those giving a result greater than 1.0 considered as positive. If the assay can be demonstrated to perform well in comparison to the Hepanostika anti-HBc Uni-Form Microelisa using matching sera it would be useful to consider expressing results as %P values - as for the RV GACELISA - to obtain a wide dynamic range and to allow for inter-assay variation. The appropriateness of the cut-off value was assessed by ROC analysis (p. 142).

RESULTS

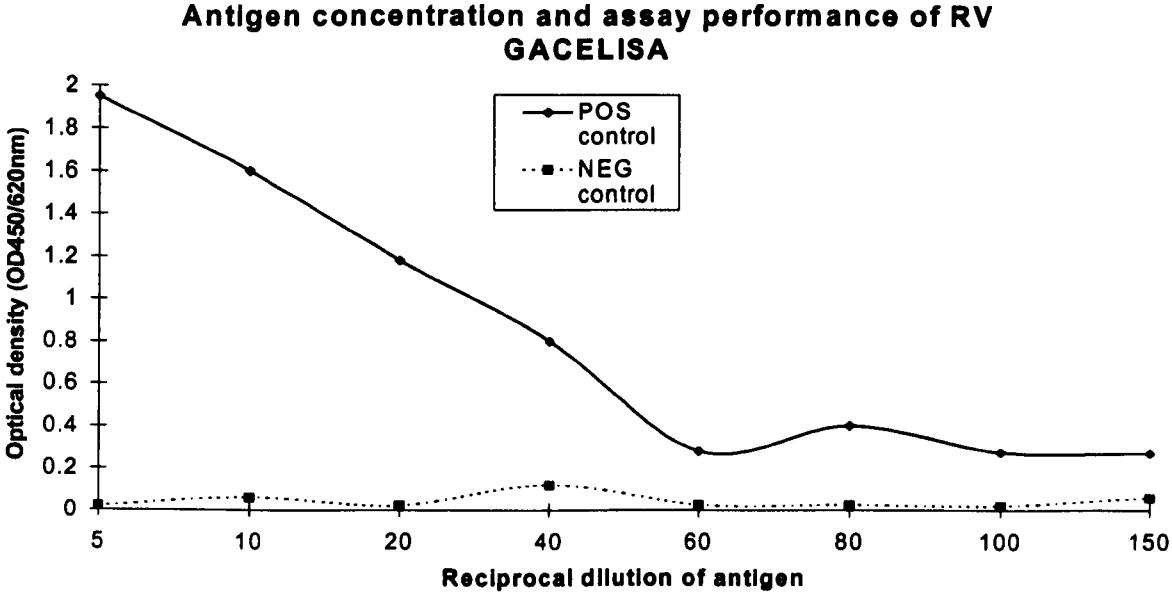
Rubella FITC/anti-FITC GACELISA.

Optimisation of test method:

Evaluation of GACELISA performance with varying RV haemagglutinin concentration

The results of the titration of RV haemagglutinin from a 1:5 to a 1:150 dilution are shown in Fig.7. These results showed that the $OD_{450/620}$ for the positive control serum decreased in a linear manner with decreasing RV haemagglutinin concentration until an RV haemagglutinin dilution of 1:60 was reached. An approximate 25% decrease in the resulting signal was obtained for each doubling dilution of RV haemaggltinin. For RV haemagglutinin dilutions greater than 1:60 the $OD_{450/620}$ remained constant. For all dilutions of RV haemagglutinin using the negative control serum the size of the signal generated remained constant giving an $OD_{450/620}$ of less than 0.1.

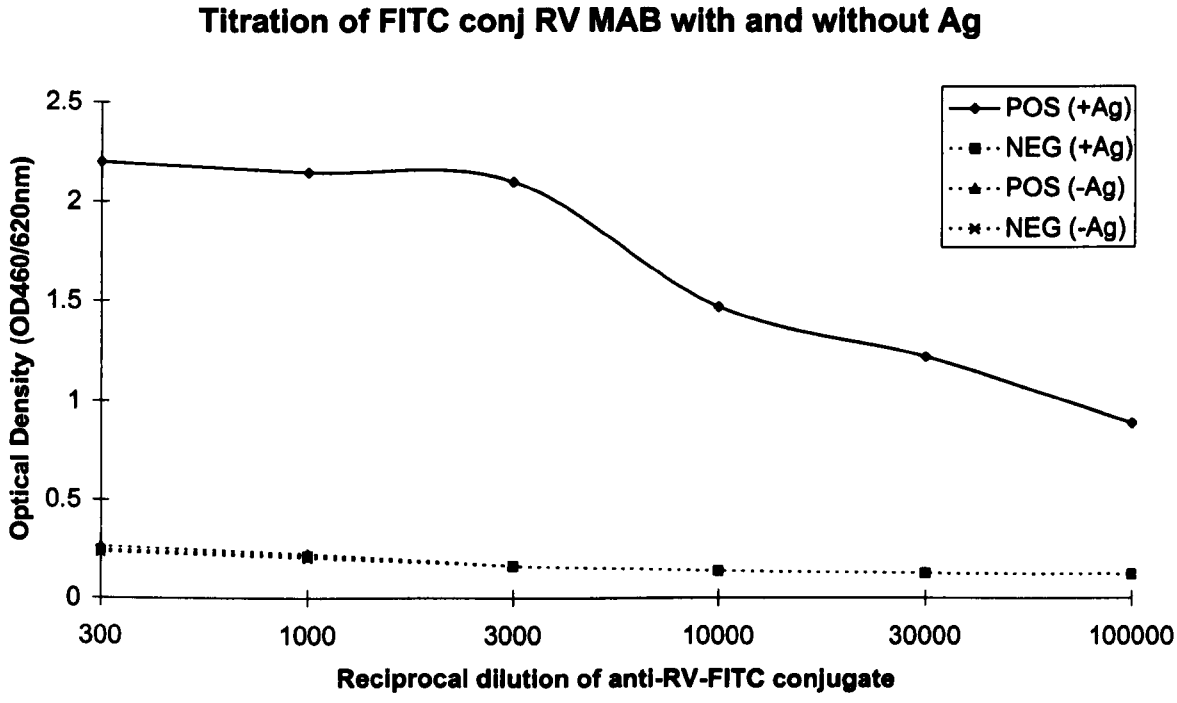
Figure 7



Determination of optimal anti-RV MAB-FITC conjugate concentration

The results of the titration of the anti-RV MAB - FITC conjugate are shown by Fig.8. Results showed that for the negative control serum in both the presence or absence of RV haemagglutinin and for the positive control serum in the absence of RV haemagglutinin, signals remained constant with an $OD_{450/620}$ of less than 0.1 for all dilutions of anti-RV MAB - FITC conjugate. When the positive control serum was used in the presence of RV haemagglutinin the size of signal generated remained approximately constant at an $OD_{450/620}$ of greater than 2.0 until dilutions of anti-RV MAB - FITC conjugate exceeded 1:3000. $OD_{450/620}$ readings then decreased with decreasing anti-RV MAB - FITC conjugate concentration.

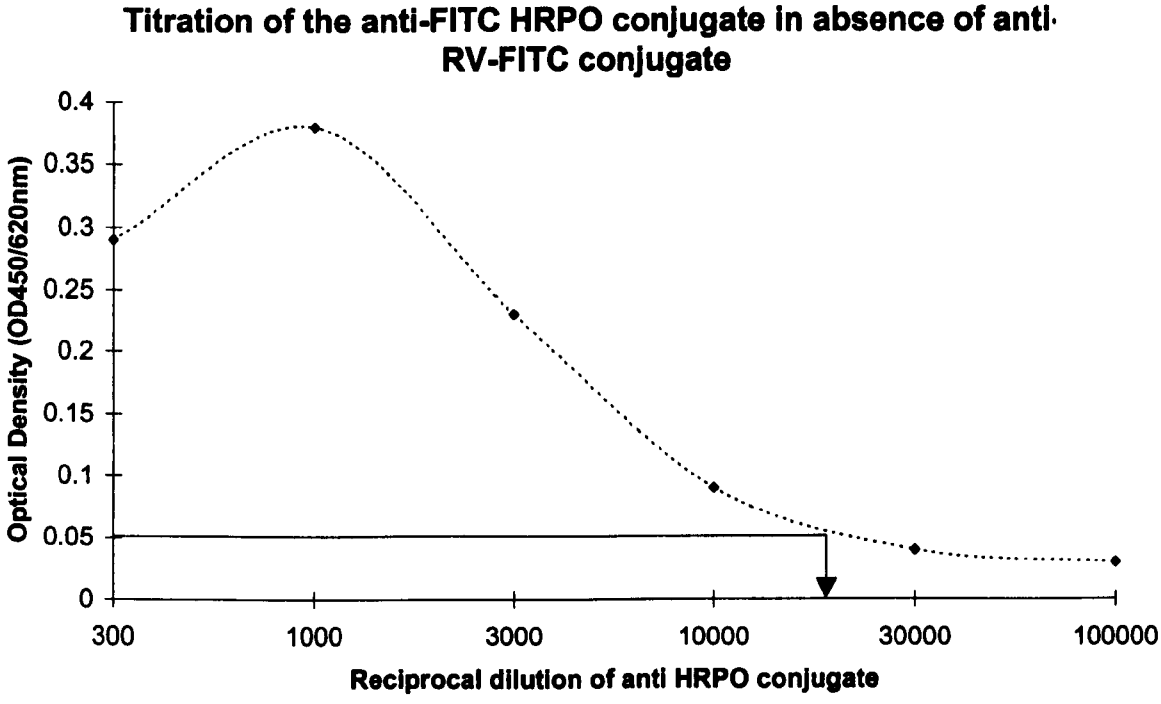
Figure 8



Determination of optimal anti-FITC HRPO conjugate concentration

An experiment was performed to investigate the level of non-specific binding by the anti-FITC HRPO conjugate. The anti-FITC HRPO conjugate was titrated and the results are shown by Fig. 9. Four positive and four negative control sera were included for each dilution of anti-FITC HRPO conjugate. Since the experiment was performed in the absence of anti-RV MAB - FITC conjugate to investigate any tendency for non-specific binding the anti-FITC HRPO conjugate may have, no difference in the size of signals for the positive and negative controls resulted. Therefore the mean combined signal was used for both the positive and negative controls at each dilution of anti-FITC HRPO conjugate.

Figure 9



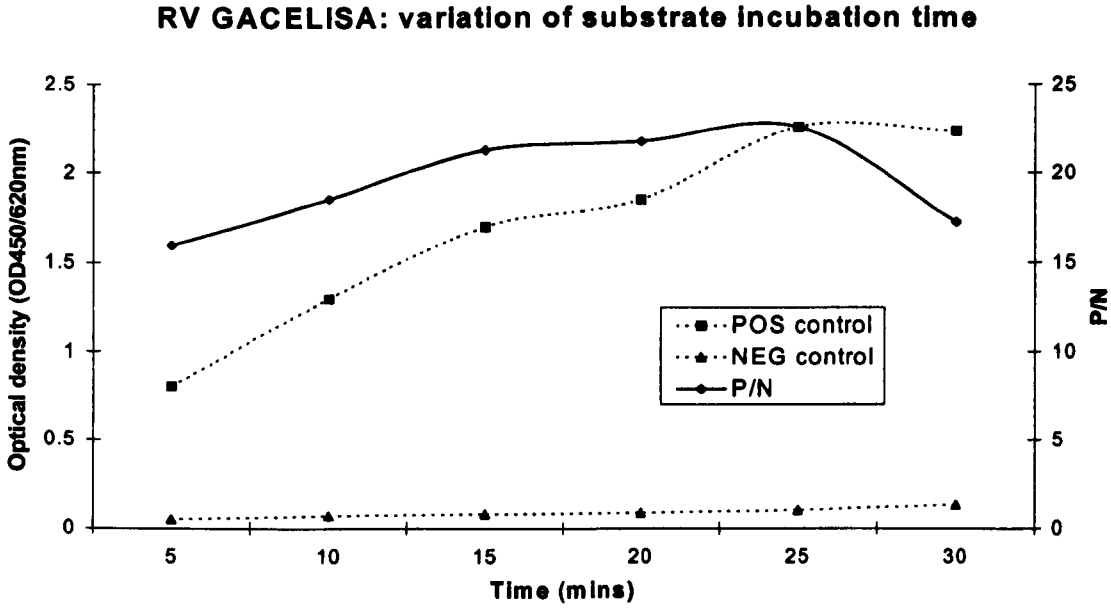
The results show that the size of signal decreased exponentially from an $OD_{450/620}$ of 0.38 to one of 0.02 as the anti-FITC HRPO conjugate was diluted down to a concentration of 1:100,000. The lowest dilution of anti-FITC HRPO conjugate where

the level of non-specific binding may be considered low enough not to significantly effect the performance of the assay ($OD_{450/620}$ ~ 0.05) indicates the optimal concentration at which it should be used (see arrow in Fig. 9).

Determination of optimal substrate incubation time

The results showing variation of optical density readings with substrate incubation time for positive and negative serum controls are shown in Fig.10. The results show that optical density readings for the positive control serum rose from 0.8 at 5 mins to 2.35 at 25 mins after which readings remained constant. For the negative control serum readings rose from 0.05 after 5 mins to 0.23 after 30 mins. A comparison of results for positive (P) and negative (N) control serum shows this ratio (P/N) to be highest at a substrate incubation time of 25 minutes.

Figure 10



A comparison of incubating shaking or stationary at 37°C

The results of titrating the WHO 80 IU/ml standard from 80 IU/ml to 0.625 IU/ml (diluted in NHS) and measuring the $OD_{450/620}$ when incubated either still or shaking, at 37°C, are shown in table 16 and Fig.11. Also shown is a comparison of the FITC/anti-FITC system to one using unconjugated mouse anti-RV MAB and an anti-mouse HRPO conjugate (see Fig. 6.).

Table 16. A comparison of assay performance when shaking and still

IU/ml	FITC/anti-FITC system		anti-mouse HRPO conjugate
	$OD_{450/620}$ Still (37°C)	$OD_{450/620}$ Shaking (37°C)	$OD_{450/620}$ Still (37°C)
80	0.36	0.41	0.270
40	0.22	0.26	0.160
20	0.14	0.21	0.150
10	0.135	0.19	0.095
5	0.09	0.13	0.085
2.5	0.07	0.1	0.075
1.25	0.07	0.095	0.07
0.625	0.08	0.08	0.07
POS control	1.251	1.412	0.950
NEG control	0.083	0.090	0.070

The results show that for the positive control serum and all dilutions of the WHO 80 IU/ml standard, with the exception of the 0.625 IU/ml dilution and for the negative control serum, $OD_{450/620}$ values were significantly higher when the microtitre plate was incubated shaking (comparison of log $OD_{450/620}$ using the FITC/anti-FITC system shaking and stationary at 37°C by paired t-test : $p < 0.05$). A similar pattern was seen when comparing the FITC/anti-FITC amplification system to that using an anti-mouse

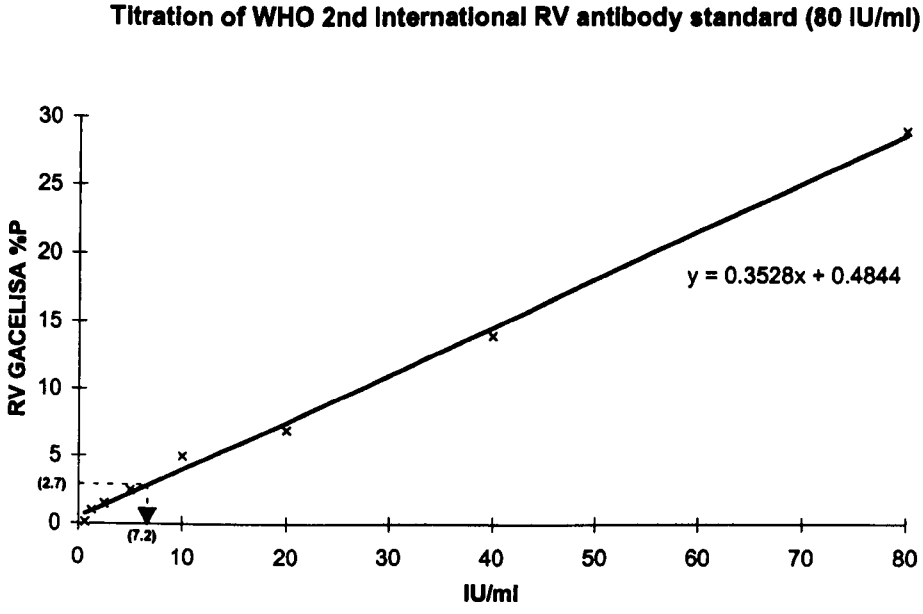
HRPO conjugate, the dynamic range of the assay being significantly higher for the FITC/anti-FITC amplification system.

Assay Performance

Assessment of assay sensitivity

A linear relationship between IU/ml and RV GACELISA %P results was observed for a titration of the WHO 80 IU/ml standard diluted in NHS. Putting the best line of fit through the points showed that the sensitivity of the RV GACELISA at the 2.7% cut-off level was 7.2 IU/ml (Fig.11).

Figure 11



Serum/oral fluid panels

The results for all three serum/oral fluid panels are shown in Table 17. Overall the amplified GACELISA showed a higher agreement than the GACRIA with the Behring ELISA by kappa and rank correlation. The exact binomial p-value of 0.011 suggests that the amplified GACELISA was significantly different to the GACRIA in comparison to the Behring ELISA. This was mainly due to the results from panel 3a and partly due to the greater correlation of the amplified GACELISA than the GACRIA with the Behring ELISA in panel 1. Compared to the Behring ELISA result on serum from all the serum/oral fluid pairs, the GACELISA was both more sensitive (82%) and specific (100%) than GACRIA, 74.4% and 97.3% respectively. The positive predictive value (PPV) for both amplified GACELISA and GACRIA was high being 100% and 99% repectively. The negative predictive value (NPV) however, was low for both oral fluid assays due to inclusion of results from panel 3a, though the NPV was higher for amplifed GACELISA (56.4%) than GACRIA (46.8%).

Panel 1: All of the 97 sera from this panel were positive for RV IgG by Behring ELISA. Of the 97 oral fluids, 92 were RV IgG positive by amplified GACELISA and 93 by GACRIA giving a sensitivity of 94.8% and 95.8% respectively. The amplified GACELISA showed a higher rank correlation with the Behring ELISA than the GACRIA. The exact binomial p-value (p=1.0), however, showed that there was no significant difference in the agreement of the two oral fluid assays with the Behring serum ELISA.

Panel 2: All 24 sera were RV IgG negative by Behring ELISA. Using the amplified GACELISA and GACRIA, the 24 corresponding oral fluids were also RV IgG negative giving a specificity of 100% for both oral fluid assays.

Panel 3a: Of 55 sera, 51 were RV IgG positive and four negative by Behring ELISA. The four oral fluids corresponding to negative sera were all RV IgG negative by both amplified GACELISA and GACRIA. Of the 51 sera which were positive, 15 corresponding oral fluids were positive by both amplified GACELISA and GACRIA, 14 positive by amplified GACELISA only and 22 negative by both assays. The sensitivity of both oral fluid assays was low compared to the Behring serum ELISA but was considerably higher for the amplified GACELISA (60.8%) than for the GACRIA (29.4%) (Table 17). The specificity and PPV was 100% for both oral fluid assays though the NPV was very low in both cases. The kappa statistic for agreement with the Behring ELISA was higher for the amplified GACELISA than for the GACRIA but low in both cases (Table 17). Rank correlation was also higher for the amplified GACELISA and the exact binomial p-value showed the amplified GACELISA agreed significantly more than the GACRIA with the Behring serum ELISA ($p= 0.0001$, Table 17).

Panel 3b: Of 21 sera, 12 were RV IgG positive and nine negative by Behring ELISA. All nine oral fluids corresponding to the nine negative sera were negative by amplified GACELISA and one oral fluid tested weakly positive by GACRIA. For the 12 sera which were positive, 10 corresponding oral fluids were positive by both assays, one negative by amplified GACELISA only and one negative by both assays. Both oral fluid assays showed a similar sensitivity (90.9%) but the specificity of the amplified GACELISA (100%) was higher than that of the GACRIA (90%) (Table 17). The PPV and NPV for both assays was high, being slightly higher for amplified GACELISA than GACRIA (Table 17). The kappa statistic was high for both assays showing good agreement with the Behring ELISA results and the rank correlation was 0.74 for both amplified GACELISA and GACRIA (Table 17). The exact binomial p-

value showed there was no significant difference in the agreement of the two oral fluid assays with the Behring serum ELISA.

Overall, from all three panels, 168 serum/oral fluid pairs gave concordant RV IgG results and 29 serum/oral fluid pairs gave discordant results. All discordant sample pairs were serum RV IgG positive by Behring ELISA and oral fluid negative by amplified GACELISA, with a geometric mean titre (GMT) of 38.3 IU/ml for serum RV IgG. Twenty two of 29 serum/oral fluid pairs showing discordant results were from panel 3a (subjects $\geq$ 17 years). For serum/oral fluid pairs with concordant positive results the serum RV IgG GMT was significantly higher, being 60.2 IU/ml (comparison of log IU/ml by t-test : $p < 0.05$).

Table 17. Comparison of the amplification based GACELISA (G) and GACRIA (R)for detection of RV-specific IgG in oral fluid with the Behring ELISA (B) with serum[a] [p. 151]

	Serum-saliva Panel				
	1	2	3a	3b	Overall
No. samples	97	24	55	21	197
Age range	3.5-4 yr	4mth-6yr	17-34 yr	5mth-10yr	4mth-34yr
Sensitivity (%):					
G	94.8	NA	60.8	90.9	82
R	95.8	NA	29.4	90.9	74.4
Specificity (%):					
G	NA	100	100	100	100
R	NA	100	100	90	97.3
PPV (%)					
G	100	NA	100	100	100
R	100	NA	100	90.9	99
NPV (%):					
G	NA	100	16.7	90.9	56.4
R	NA	100	10	90	46.8
No. samples with the following result[b]:					
B+, G+, R+	89	0	15	10	114
B+, G+, R-	3	0	14	0	17
B+, G-, R+	4	0	0	1	5
B+, G-, R-	1	0	22	1	24
B-, G-, R+	0	0	0	1	1
B-, G-, R-	0	24	4	9	37
Kappa statistic (95% CI) for agreement with B[c]:					
G	NA	NA	0.16 (0.01-0.31)	0.81 (0.57-1.00)	0.63 (0.51-0.74)
R	NA	NA	0.06 (-0.01-0.12)	0.90 (0.72-1.00)	0.51 (0.39-0.62)
Rank correlation (95% CI) with B:					
G	0.74 (0.63-0.82)	0.21 (-0.21-0.56)	0.58 (0.37-0.73)	0.74 (0.44-0.88)	0.68 (0.60-0.75)
R	0.55 (0.40-0.68)	-0.06 (-0.45-0.35)	0.35 (0.10-0.57)	0.74 (0.46-0.89)	0 .45 (0.33-0.56)
Exact binomial *P* value[d]	1.00	1.00	0.0001	1.00	0.011

(Footnotes overleaf)

[a] G = amplification -based GACELISA; R = GACRIA; B = Behring ELISA; NA = not applicable; CI = confidence interval; + = positive result; - = negative result

[b] No samples fell into the categories B-,G+,R+ or B-,G+,R-

[c] The coefficient of agreement of the kappa statistic is based on comparison of the results of the Behring ELISA with those of the amplification-based GACELISA and the results of the Behring ELISA with those of GACRIA. It can be calculated only when both samples are positive and negative by the Behring ELISA

[d] The *P* value in effect compares samples with B+,G+,R- and B-,G-,R+ results with samples with B+,G-,R+ results, which are the totals when the results of one method agree with those of the Behring ELISA and the results of the other method do not.

Screening a rural Ethiopian population for rubella seroprevalence

The FITC/anti-FITC GACELISA was used to screen 831 oral fluid samples collected by Oracol from a rural Ethiopian population living in the Butajira region. Matching serum samples for all oral fluids were collected and screened for rubella specific IgG using a commercial ELISA (Behring). The results are shown in Table 18, 19 and Fig. 12. Rubella seroprevalence, as measured by Behring, rose with age from 27% in those aged 0-4 years to 100% in persons aged >20 years. A comparison of oral fluid and serum results showed that the sensitivity of the oral fluid GACELISA relative to serum ELISA varied with the age of subject, ranging from 92% in persons aged 5-9 years to 69% in those >50 years, and showed a generally decreasing trend with age. The specificity of the oral fluid assay also varied according to age ranging from 89.6% in persons aged 0-4 years to 100% in persons aged >30 years. Overall, when screening oral fluid samples from a rural Ethiopian population, the sensitivity and specificity of the oral fluid GACELISA, relative to serum ELISA, was 79% and 90% respectively.

Table 18. The seroprevalence of rubella in Butajira (Ethiopia), 1997

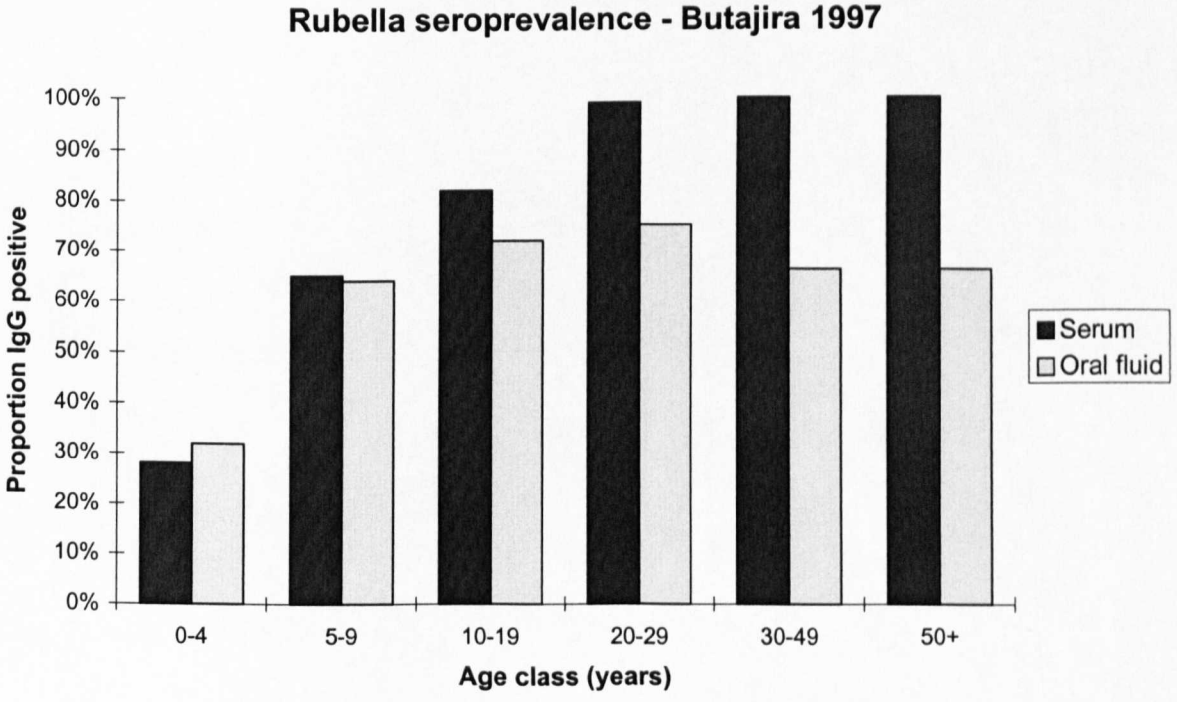
Age Grp	SERUM		Oral Fluid	
(yrs)	NEG (%)	POS (%)	NEG (%)	POS (%)
0-4	67 (70.5)	28 (29.5)	64 (67.4)	31 (32.6)
5-9	53 (34.4)	101 (65.6)	56 (36.4)	98 (63.8)
10-19	42 (17.3)	201 (82.7)	64 (26.3)	179 (73.7)
20-29	1 (1.6)	63 (98.4)	14 (21.9)	50 (78.1)
30-49	1 (0.9)	167 (99.1)	57 (33.9)	111 (66.1)
50+	0 (0)	107 (100)	33 (30.8)	74 (69.2)
TOTAL	164 (19.7)	667 (80.3)	288 (34.7)	543 (65.3)

Table 19. Age specific sensitivity, specificity, NPV and PPV of the RV GACELISA on oral fluid samples relative to Behring Enzygnost results on matching sera.

Age Group (Years)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0-4	85.7	89.6	77.4	93.8
5-9	92.1	90.6	94.9	85.7
10-19	87.1	90.5	97.8	59.4
20-29	77.8	NA*	98.0	0
30-49	66.5	NA*	100	1.8
50+	69.2	NA*	100	0
TOTAL	**78.9**	**89.6**	**96.9**	**51.0**

*Not appropriate to be calculated as the majority of serum samples for persons aged >20 years were positive for RV specific IgG by Behring Enzygnost.

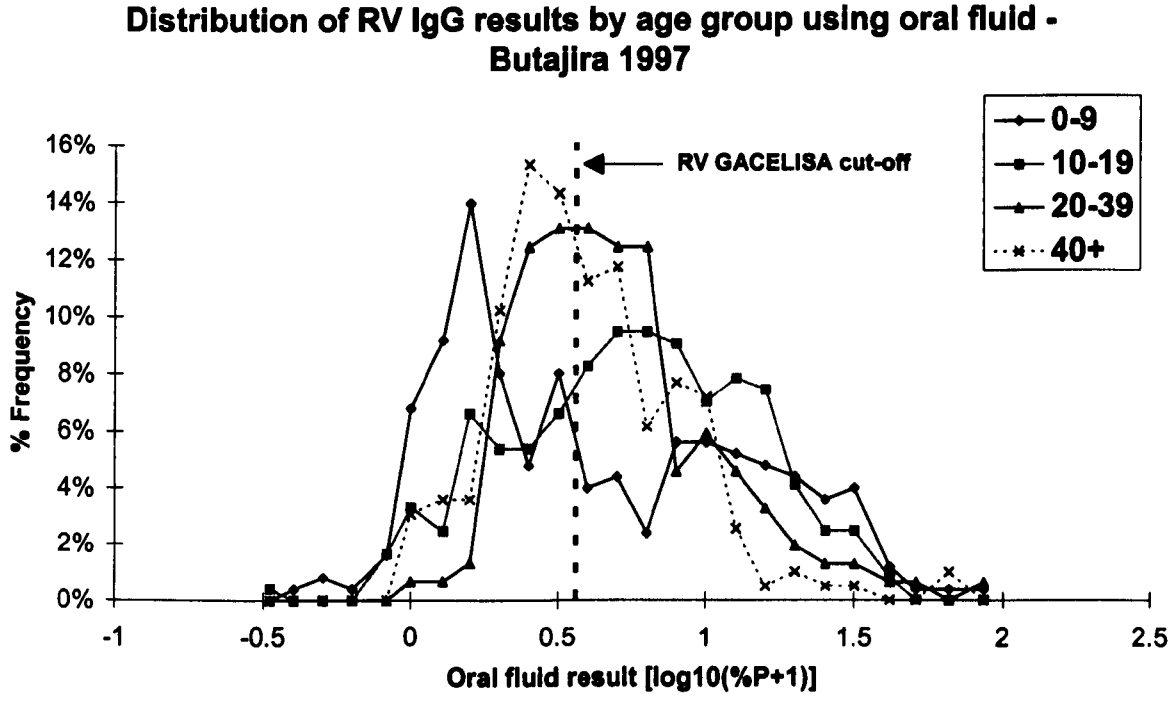
Figure 12



Distribution of RV IgG results, Butajira 1997

The distribution by age group for the 831 oral fluid samples obtained from the rural Ethiopian population that were screened for RV IgG antibody by GACELISA is shown in Fig. 13. The perpendicular broken line represents the RV GACELISA cut-off value. The distribution of results changed with increasing age of the subjects, results shifting to the right of the assay cut-off for subjects aged ≥10 years. A particularly clear distinction was made between those aged 0-9 years and those aged 10-19 years, whilst it was not so easy to distinguish between results from those aged >20 years and those aged 0-9 years.

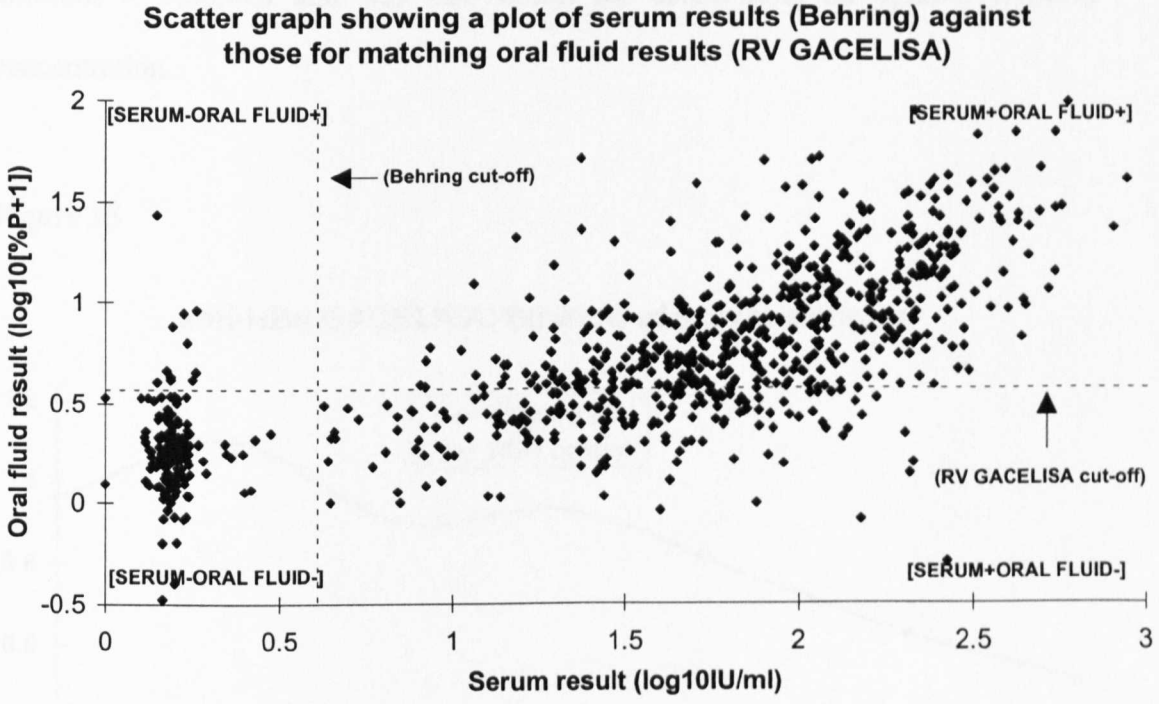
Figure 13



The distribution of oral fluid results using the RV GACELISA in comparison to matching serum results (Behring) with respect to each of the assay cut-offs is shown

in Fig.14. This clearly shows the ability of the Behring ELISA to categorise sera into those that are positive and negative for RV IgG which cannot be done to the same extent using oral fluid samples with the RV GACELISA. The broken lines represent the serum and oral fluid assay cut-offs and divide the graph into four sections that may be used to categorise agreement between results for the two assays. Those falling in the bottom right hand section of the graph represent those oral fluid samples which tested negative in the RV GACELISA but with matching sera which tested positive by Behring.

Figure 14



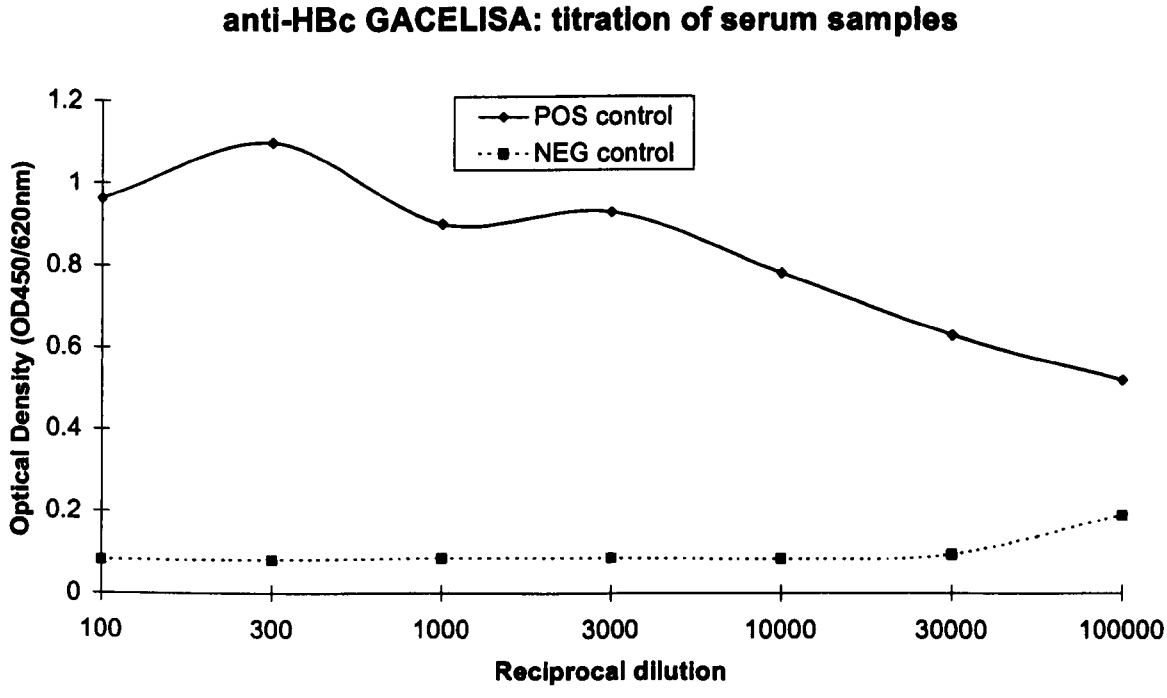
Anti-HBc GACELISA

Optimisation of assay:

Titration of serum samples

Positive and negative control sera for anti-HBc were titrated (Fig.15). The $OD_{450/620}$ for the positive control decreased with increasing serum dilution. The signal for the negative control remained constant at an $OD_{450/620}$ of < 0.1 until a dilution of >1:30,000 had been reached where the signal began to rise. The positive control serum was also titrated in conjunction with rHBcAg (Fig. 16) which showed that the greatest decrease in $OD_{450/620}$ of the positive control serum was seen when using serum dilutions > 1:30,000 and was also useful for determining an optimal rHBcAg concentration.

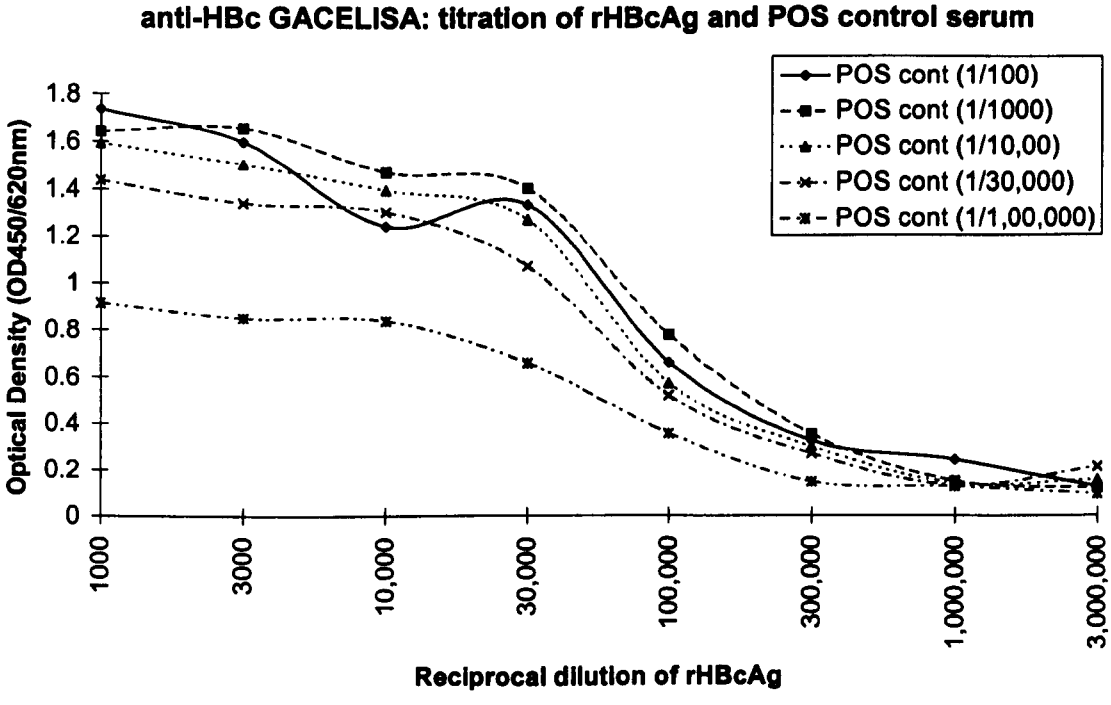
Figure 15



Titration of rHBcAg

A titration of the rHBcAg showed that the more concentrated this reagent was used the higher the resulting $OD_{450/620}$ for the positive control serum (Fig. 16). This was also proportional to the concentration of positive control serum used. The $OD_{450/620}$ for the positive control serum decreased markedly when dilutions of rHBcAg exceeded a factor of 1:30,000. For reasons of economy a rHBcAg dilution of 1:3000 was chosen, though the development of an assay with a greater dynamic range would be possible if the rHBcAg could be used at a higher concentration.

Figure 16

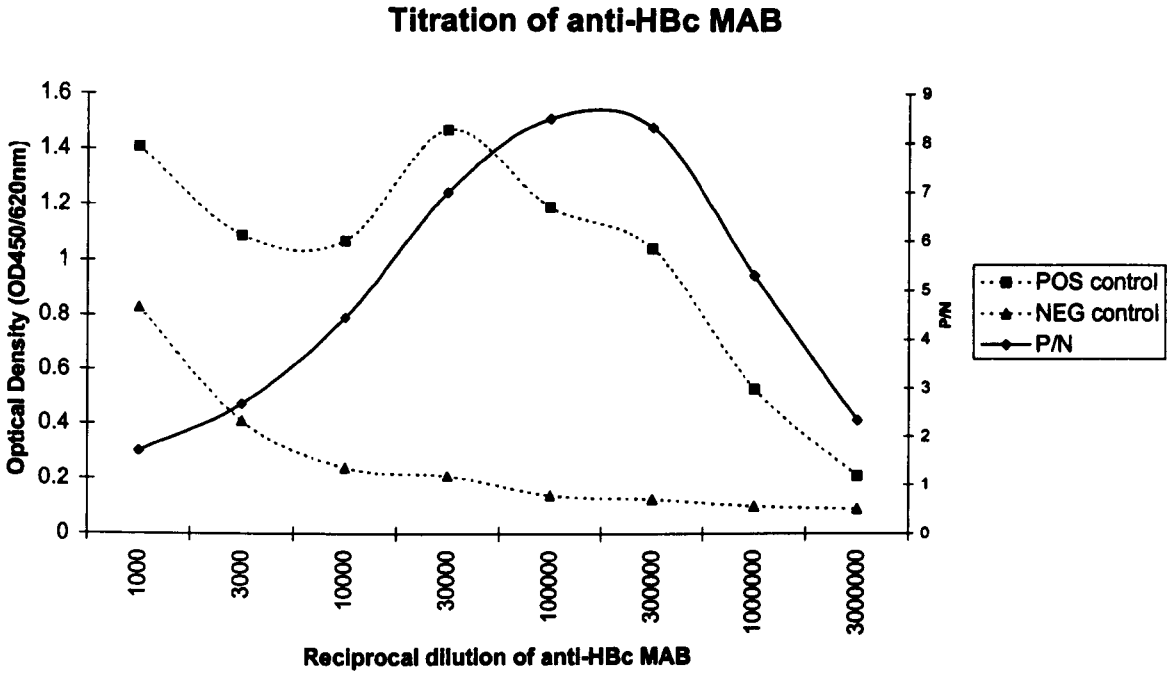


Mouse anti-HBc MAB

A titration of mouse anti-HBc MAB using positive and negative serum controls is shown in Fig. 17. The observed trend shows a decrease in $OD_{450/620}$ readings for both positive and negative control sera with increasing dilution of mouse anti-HBc MAB, though were considerably lower for the negative control (the increase in $OD_{450/620}$ for the positive control serum between anti-HBc MAB dilutions of 1/10,000 and 1/30,000 may be a result of a dilution error or may be due to a "prozone" effect (145). At dilutions of $\geq$ 1:100,000 for the MAB, $OD_{450/620}$ readings for the negative control were < 0.1. A comparison of signals for the positive (P) and negative (N) controls showed the P/N ratio to be highest at a MAB dilution of 1:30,000. However, at this dilution the $OD_{450/620}$ reading for the negative control was > 0.1.

That $OD_{450/620}$ readings for the negative control decreased with increasing dilution factor of anti-HBc MAB (from ~0.8 to > 0.1) suggested this reagent had a tendency to bind non-specifically to other components of the assay. It was therefore important to choose a dilution of MAB which gave the greatest dynamic range and a low level of non-specific binding ($OD_{450/620} < 0.1$ for the negative control). A dilution of 1/100,000 for the anti-HBc MAB was therefore chosen.

Figure 17



Anti-mouse HRPO

Titrating the anti-mouse HRPO using positive and negative control sera showed a similar pattern to the parallel experiment involving the mouse anti-HBc MAB : signals for both the positive and negative control sera decreased with increasing dilution of anti-mouse HRPO (Fig. 18). Signals for the negative control dropped below an $OD_{450/620}$ of 0.1 for anti-mouse HRPO dilutions $\geq$ 1:4000 and a comparison of $OD_{450/620}$ readings for the positive and negative controls showed the P/N ratio to be greatest using an anti-mouse HRPO dilution of 1:4000. A closer examination of the anti-mouse HRPO was made using dilutions ranging from 1:3500 to 1:5000 (Fig.19) showed that the assay had the greatest dynamic range using the anti-mouse HRPO at a dilution of 1:3500.

Figure 18

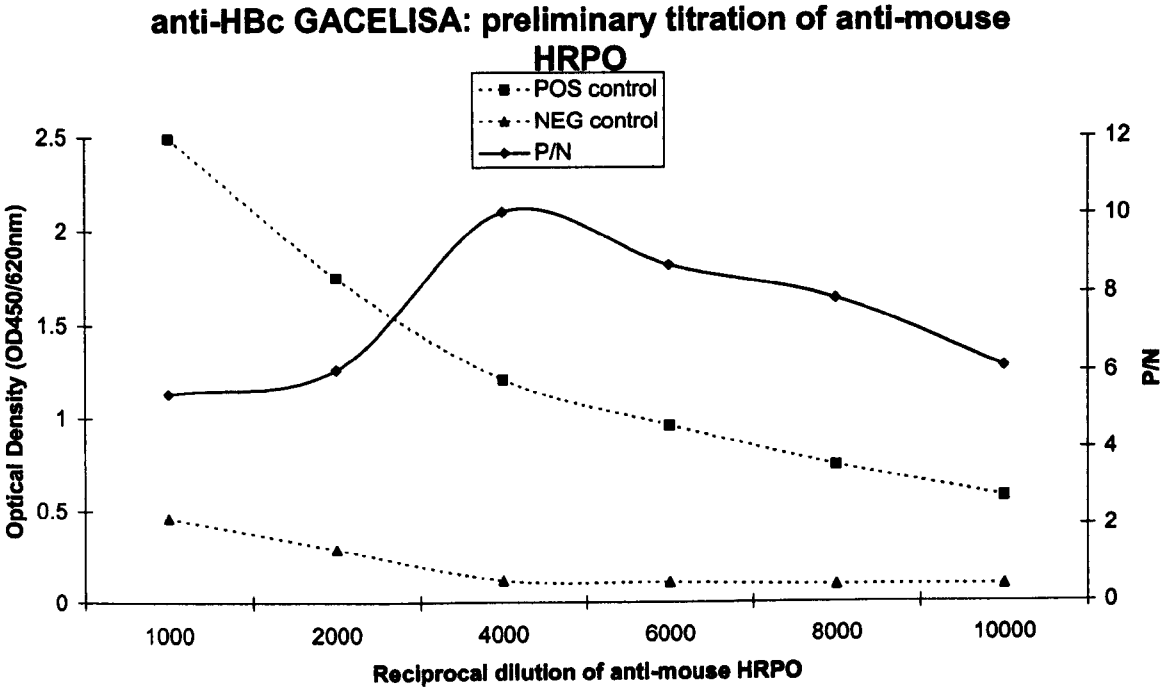
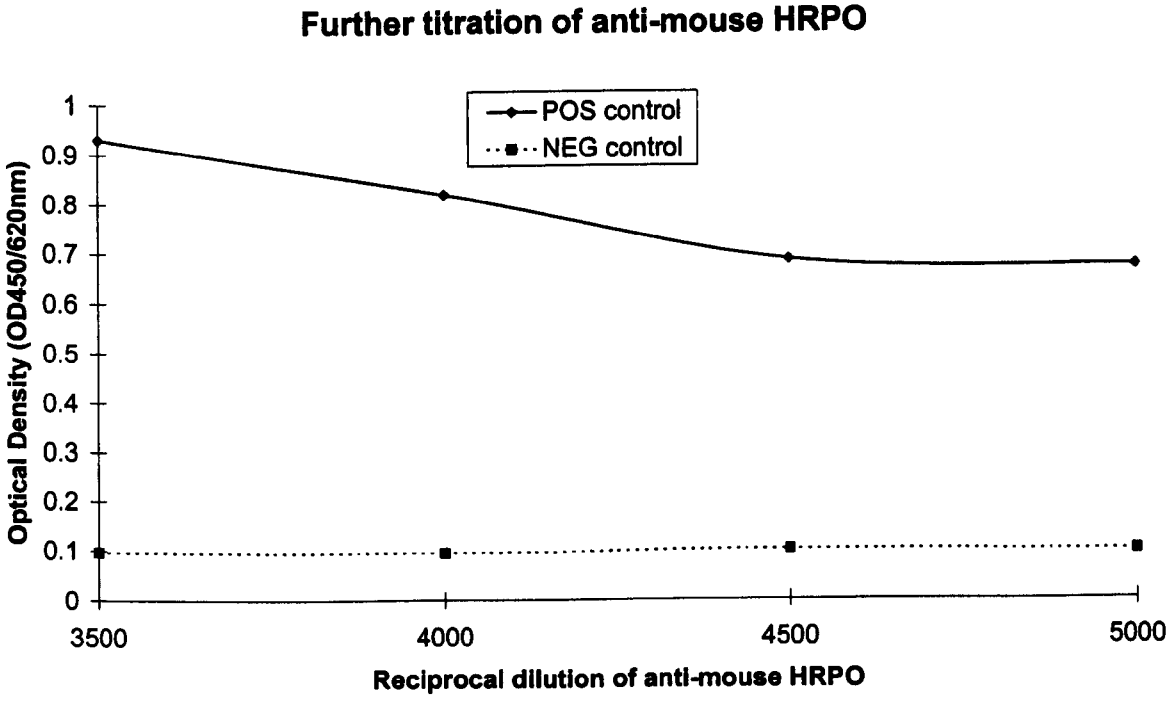


Figure 19



Evaluation of assay performance

Non-specific binding

To investigate the cause of non-specific binding highlighted by results shown in Fig 17, an experiment was performed where individual components were omitted from the assay as shown in Table 20.

Table 20. Investigating the cause of non-specific binding.

Row No.	Reagents Included					$OD_{450/620}$
1	anti-gamma	neg control	Ag	MAB	CONJ	**0.097**
2	anti-gamma	pos control	Ag	MAB	CONJ	**1.862**
3	anti-gamma	---	Ag	MAB	CONJ	**0.255**
4	anti-gamma	---	---	MAB	CONJ	**0.288**
5	anti-gamma	---	---	---	CONJ	**0.05**
6	anti-gamma	pos control	---	MAB	CONJ	**0.111**
7	anti-gamma	pos control	---	---	CONJ	**0.112**

These results showed that the anti-HBc monoclonal antibody tended to bind non-specifically to any available anti-gamma bound to the solid phase (Row 3 and 4). This did not occur if a positive or negative control had already been added (Rows 1, 2 and 6). The conjugate showed no evidence of non-specific binding in the absence of anti-HBc MAB (Rows 5 and 7).

To help overcome non specific binding it was decided to examine the effect of including 2% NHS in the anti-HBc monoclonal antibody diluent. The results are shown in table 21.

Table 21. The effect of including 2% NHS in the anti-HBc monoclonal antibody diluent.

Row No.	Reagents Included					$OD_{450/620}$
1	anti-gamma	NHS	Ag	MAB	CONJ	**0.089**
2	anti-gamma	NHS	Ag	MAB(NHS)	CONJ	**0.115**
3	anti-gamma	pos control	Ag	MAB	CONJ	**2.057**
4	anti-gamma	pos control	Ag	MAB(NHS)	CONJ	**1.869**
5	anti-gamma	---	Ag	MAB	CONJ	**0.317**
6	anti-gamma	---	Ag	MAB(NHS)	CONJ	**0.115**
7	anti-gamma	---	---	MAB	CONJ	**0.312**
8	anti-gamma	---	---	MAB(NHS)	CONJ	**0.102**

Rows 1-4 show that the inclusion of 2% NHS in the monoclonal antibody diluent did not significantly affect the $OD_{450/620}$ readings obtained using the control samples. Rows 6 and 8 show the addition of 2% NHS in the monoclonal antibody diluent reduced the absorbance of the signal obtained to a value comparable to that of the negative control sample. If the 2% NHS was not included the absorbance values of the resulting signals were considerably higher (Rows 5 and 7).

Anti-HBc in blood donor sera: results of GACELISA and Hepanostika ELISA

A total of 23 blood donor sera were tested by both Hepanostika anti-HBc Uni-Form Microelisa system (ORGANON Teknika) and anti-HBc GACELISA (Table 22). Six sera (sera 1-6) were positive both by the Hepanostika kit and the anti-HBc GACELISA. To simulate the low concentrations of anti-HBc expected in oral fluids, serum samples were diluted from 1:100 to 1:10,000. Serum diluted 1:1000 has IgG antibody concentrations approximating those found in oral fluid (Table 3). Sera 1-5 were still anti-HBc positive by GACELISA when diluted 1:10,000 whilst serum 6 was positive only at a dilution of 1:100.

Table 22. A comparison of results from sera tested by anti-HBc GACELISA and Organon (Hepanostika anti-HBc Uni-Form) kit.

Serum No.	Organon Result	anti-HBc GACELISA ($OD_{450/620}$) ['working' cut off $OD_{450/620}$ = 0.150]			
		Dilution Factor			
		1:100	1:1000	1:3000	1:10,000
1	POS	0.214	0.194	0.204	0.177
2	POS	1.083	0.969	1.013	0.827
3	POS	2.918	2.540	2.700	2.330
4	POS	0.762	0.650	0.704	0.624
5	POS	1.151	0.920	1.098	0.903
6	POS	0.432	0.127	0.122	0.125
7	POS	0.104	0.129	0.102	0.121
8	POS	0.106	0.111	0.127	0.107
9	POS	0.078	0.076	0.076	0.075
10	POS	0.106	0.099	0.107	0.097
11	POS	0.101	0.082	0.087	0.138
12	POS	0.130	0.086	0.084	0.082
13	POS	0.106	0.110	0.086	0.083
14	POS	0.086	0.091	0.091	0.082
15	NEG	0.087	0.081	0.092	0.096
16	NEG	0.087	0.088	0.085	0.093
17	NEG	0.077	0.071	0.073	0.086
18	NEG	0.101	0.120	0.104	0.087
19	NEG	0.088	0.094	0.092	0.091
20	NEG	0.089	0081	0.117	0.088
21	NEG	0.078	0.087	0.082	0.086
22	NEG	0.082	0.103	0.088	0.081
23	NEG	0.090	0.120	0.222	0.095

Sera 7-14 gave positive results using the Hepanostika system but were negative using GACELISA at all serum dilutions used. Sera 15-23 gave negative results using the Hepanostika kit and were all also negative at all dilutions using GACELISA.

Titration of four oral fluid samples from persons of known anti-HBc status

Four oral fluids, two from persons confirmed as anti-HBc IgG positive and two from persons anti-HBc IgG negative, were titrated and tested by anti-HBc GACELISA (Table 23).

Table 23. Results for 4 oral fluids tested by anti-HBc GACELISA.

Dilution	anti-HBc GACELISA OD $_{450/620}$ ['working' cut off OD $_{450/620}$ = 0.150]			
	Oral fluid 1	Oral fluid 2	Oral fluid 3	Oral fluid 4
Undiluted	1.385	1.853	0.127	0.119
1/2	1.140	1.325	0.104	0.124
1/4	1.045	0.924	0.090	0.112
1/8	0.944	0.683	0.108	0.115
1/16	0.878	0.445	0.110	0.130
1/32	0.642	0.362	0.105	0.117

Oral fluids 1 and 2 were collected from persons confirmed as being anti-HBc IgG positive and gave strong signals with high absorbance values when tested undiluted by GACELISA and still tested positive when diluted 1/32. The $OD_{450/620}$ readings given by oral fluid 1 when tested at 1/32 was approximately half that when tested undiluted. When oral fluid 2 was diluted 1/32 the absorbance value was approximately 5 times lower than when tested undiluted. Oral fluids 3 and 4 were collected from persons known to be anti-HBc negative and both gave $OD_{450/620}$ readings low enough to be considered negative for anti-HBc at all dilutions tested.

Screening oral fluid samples

A total of 334 oral fluid samples collected by the salivette device, were tested for total anti-HBc by an adaption of the ICE HBc Detection Pack (Murex Diagnostics Limited, UK), and those positive tested by GACRIA. These tests were carried out by staff at the Hepatitis and Retrovirus Laboratory (HRL), CPHL, and the results kindly made available. The samples were then tested by GACELISA (Table 24).

Table 24. A comparison of the ICE HBc Detection Pack and the anti-HBc GACELISA on oral fluid samples (n = 334)

	ICE HBc +	ICE HBc -
anti-HBc GACELISA +	**130**	**18**
anti-HBc GACELISA -	**18**	**168**

Performance of anti-HBc GACELISA compared to "ICE"

Sensitivity = 88% (130/148)

Specificity = 90.3% (168/186)

Positive Predictive value = 88% (130/148)

Negative Predictive value = 90.3% (168/186)

Of the oral fluids testing positive by the Murex ICE test, 10 were negative by anti-HBc GACRIA with one of these testing positive in the anti-HBc GACELISA. Of the 18 oral fluids which were positive in the Murex ICE test but negative by anti-HBc GACELISA, all were weakly positive by anti-HBc GACRIA (mean T/N value = 3.7). Eighteen samples were negative by the Murex ICE test but positive by GACELISA.

Fifteen had sufficient volume for testing by GACRIA, of which 2 were positive, 1 equivocal and 12 negative.

Serum/Oral fluid Pairs

A total of 538 serum/oral fluid pairs were obtained from a rural Ethiopian population. The sera were screened for anti-HBc using the Hepanostika anti-HBc Uni-Form Microelisa system and the corresponding oral fluid samples tested for anti-HBc IgG antibody by GACELISA. An analysis af anti-HBc GACELISA performance compared to Hepanostika relative to age of subject is shown in Table 25, 26 and Fig.20.

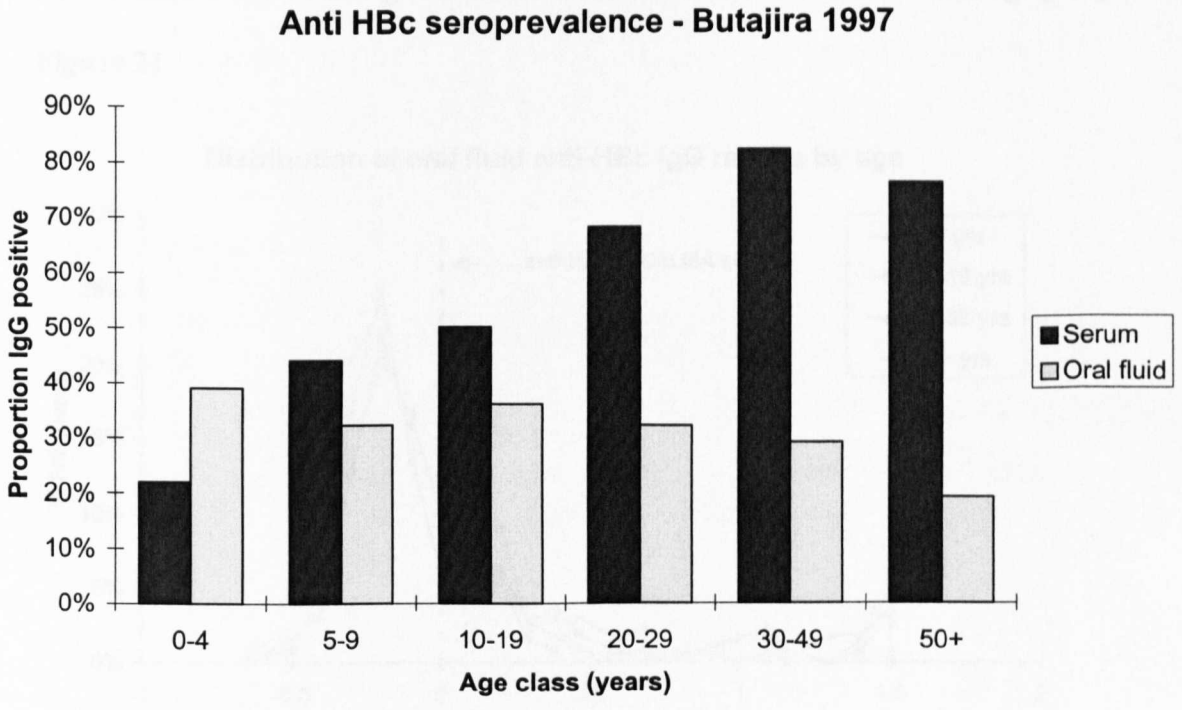
Table 25. The seroprevalence of anti-HBc in Butajira (Ethiopia), 1997

Age Group (years)	Serum		Oral Fluid	
	NEG (%)	POS (%)	NEG (%)	POS (%)
0-4	35 (79.5)	9 (20.5)	27 (61.4)	17 (38.6)
5-9	52 (60.5)	34 (39.5)	58 (67.4)	28 (32.6)
10-19	75 (49.0)	78 (51.0)	98 (64.1)	55 (35.9)
20-29	16 (34.0)	31 (66.0)	33 (70.2)	14 (29.8)
30-49	21 (16.9)	103 (83.1)	88 (71.0)	36 (29.0)
50+	17 (21.8)	61 (78.2)	64 (82.1)	14 (17.9)
TOTAL	216 (40.6)	316 (59.4)	368 (69.2)	164 (30.8)

Table 26. Age specific sensitivity, specificity, NPV and PPV of the anti-HBc GACELISA on oral fluid samples relative to Organon results on matching sera (samples: Butajira, Ethiopia 1997).

Age Group (Years)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
0-4	66.7	68.6	35.3	88.9
5-9	67.7	90.4	82.1	81.0
10-19	57.7	86.7	81.8	66.3
20-29	41.9	93.8	92.9	45.5
30-49	33.0	90.5	94.4	21.6
50+	23.0	100.0	100.0	26.6
TOTAL	**42.7**	**86.6**	**82.3**	**50.8**

Figure 20



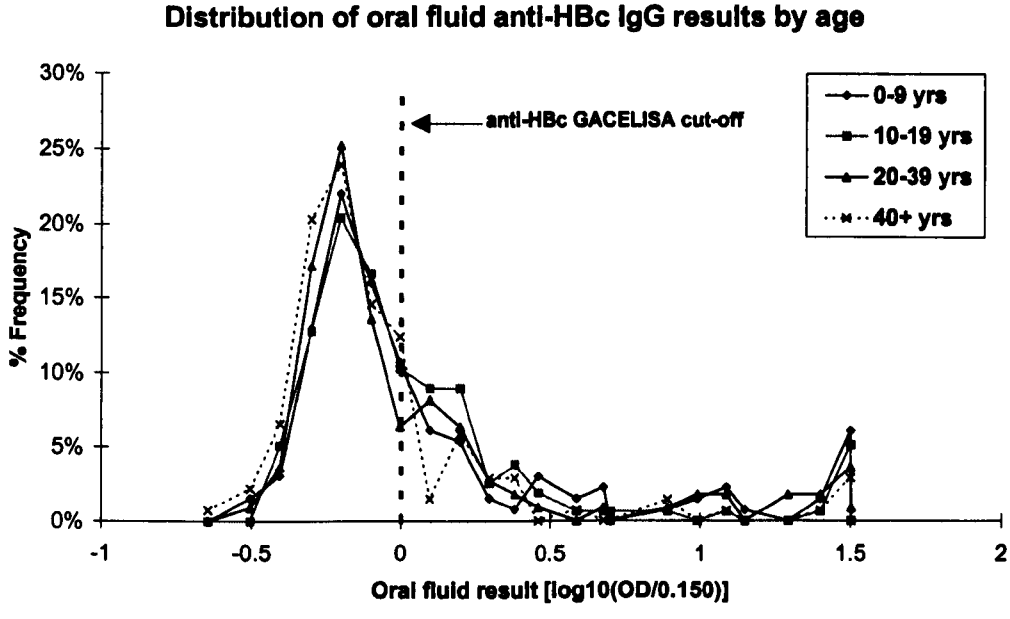
The results show that anti-HBc seroprevalence, as measured by Hepanostika, rose with age from 22% in persons aged 0-4 years to 80% in those aged >30 years. The

analysis of oral fluid results shows a general decrease in anti-HBc GACELISA sensitivity with increasing age of subject, ranging from 67% in the 0 - 9 year old group to 21% in persons aged >50 years. In contrast the specificity of the anti-HBc GACELISA increased with the age of subjects from 69% in the 0-4 year old group to 100% in those aged > 50 years. Overall the anti-HBc GACELISA showed a sensitivity and specificity, relative to Hepanostika, of 43% and 87% respectively.

Distribution of anti-HBc IgG results, Butajira 1997

The distribution of results by age group for the 530 oral fluid samples obtained from the rural Ethiopian population that were screened for anti-HBc IgG antibody by GACELISA is shown in Fig 21. Similar distributions were found for each age group.

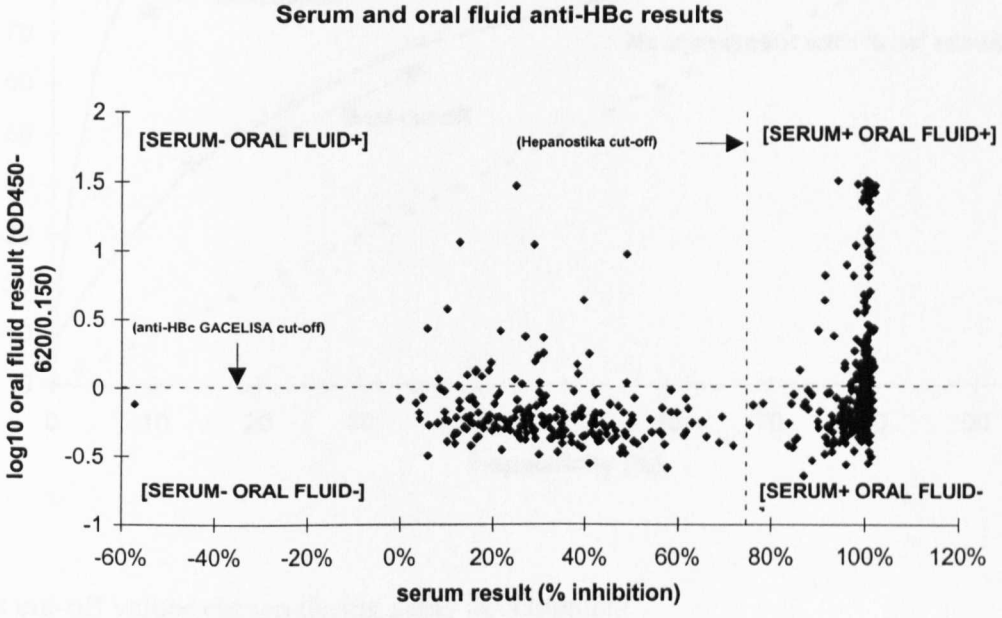
Figure 21



The distribution of oral fluid results using the anti-HBc GACELISA compared to matching serum results (Hepanostika) with respect to each of the assay cut-off values is shown in Fig. 22. As was the case with the assays used to screen for RV specific

IgG, this clearly showed the ability of the Hepanostika ELISA to categorise sera into those that are positive and negative for anti-HBc which is not reflected in the performance of the anti-HBc GACELISA using oral fluid. As for the RV study, the graph was divided into four sections, determined by the respective cut-off values for the anti-HBc GACELISA and Hepanostika ELISA, illustrating the agreement of results from each assay. The large proportion of false negative anti-HBc GACELISA results can be seen in the bottom right hand section of Fig. 23.

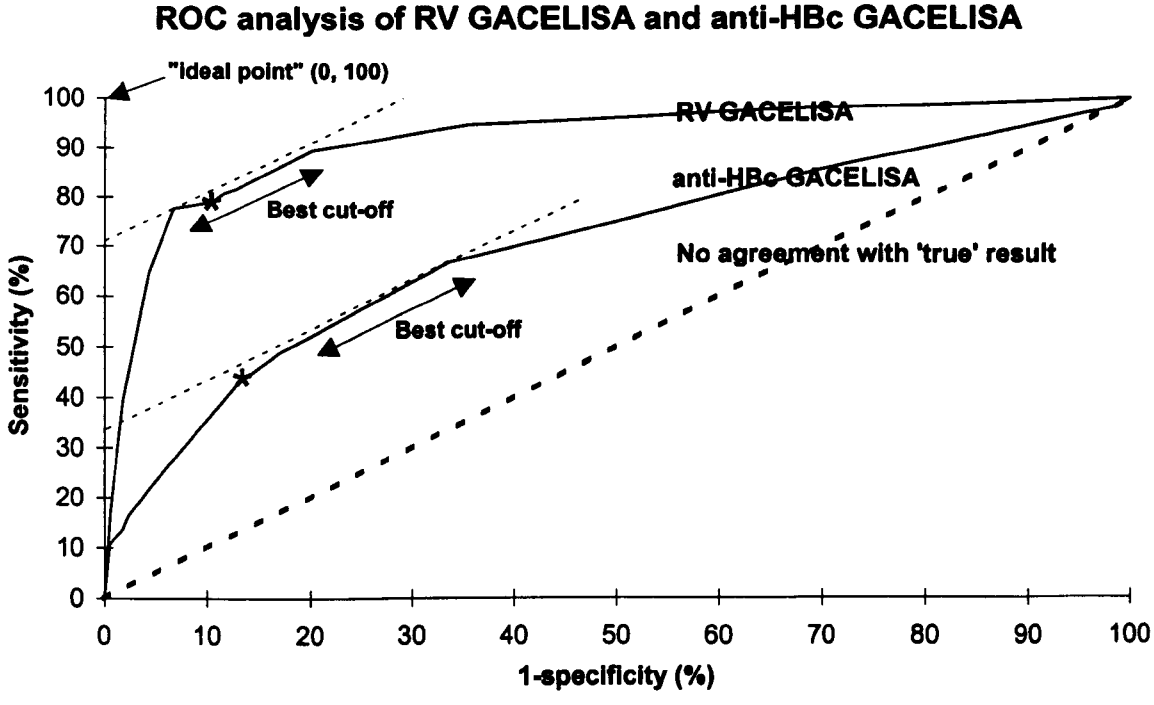
Figure 22



ROC analysis of RV GACELISA and anti-HBc GACELISA

ROC curves using a range of assay cut-off values were constructed for the RV GACELISA and anti-HBc GACELISA (Fig. 23).

Figure 23



* cut-off values chosen during assay development

Also included in Fig. 23 are the cut-off values chosen during assay development, the ideal point of (0, 100) which represents 100% sensitivity and specificity with respect to the gold standard assay, and a line representing no agreement with the gold standard assay. The flatness of the curve for both the RV GACELISA and anti-HBc GACELISA around the region where the gradient is 1.0 makes it difficult to select an exact point which indicates the cut-off where sensitivity is maximised to a level of

minimum effect on specificity. Therefore a region denoting where this point is likely to fall is shown rather than the precise point itself. The working cut-off value used for the RV GACELISA was located in this region but that used for the anti-HBc GACELISA was below that indicated by ROC analysis.

DISCUSSION

Development of oral fluid assays for virus specific IgG antibody

Having compared and evaluated three oral fluid collection devices two ELISA assays were developed. These were designed specifically for use with oral fluid samples in basic laboratories in developing countries. However, further simplification is needed if the assays are to be used successfully under field conditions. Since oral fluid contains antibody at a considerably lower concentration than that found in serum, it was important that the assay format used provided a system that would be sufficiently sensitive. An antibody capture format was chosen as the most suitable format for assay development for two reasons:

- antibody capture assays measure the proportion of specific antibody in the total antibody present in a sample rather than the concentration of specific antibody. This proportion will, theoretically, remain constant at all sample dilutions so the proportion of specific IgG found in a oral fluid should reflect that found in serum.
- the anti-human IgG antibody used to coat the solid phase to "capture" the IgG present in a sample becomes saturated at low IgG concentrations. Therefore antibody capture assays are particularly suitable for use with oral fluid samples, where the antibody concentration is expected to be low (hence avoiding the need to standardise for total IgG concentrations in oral fluid samples).

Assays were developed with the aim of studying the epidemiology of two vaccine preventable virus infections, rubella and hepatitis B, in rural communities in developing countries. Both assays were used to screen samples collected from a rural Ethiopian population.

Rubella FITC/anti-FITC GACELISA

Assay development

In order to develop assays for oral fluid RV IgG that could be more widely used than the previously described GACRIA (144) it was decided to develop a corresponding IgG capture ELISA test. Initial studies (Table 16) showed that simply substituting an HRPO conjugate for the ^{125}I conjugated antibody used in radioimmunoassays did not result in an ELISA with sufficient sensitivity so it was decided to use the FITC/anti-FITC amplification system (165). A comparison with the non-amplified assay showed the FITC/anti-FITC amplification system produced an ELISA with a considerably larger dynamic range enabling a better discrimination between positive and negative results. The improvement in assay performance using the FITC/anti-FITC amplification system is a result of using a second monoclonal antibody directed against FITC, several molecules of which are conjugated to each RV MAB molecule. This serves to enhance sensitivity. Also, since FITC is a molecule not found in naturally occuring biological samples, it would not be involved in non specific reactions involving other assay reagents so helping to improve assay specificity.

An alternative amplification system that could have been chosen was that which employs the use of biotin and streptavidin conjugated reagents. The biotin-streptavidin interaction has an extremely high affinity and has been used in a wide variety of diagnostic tests. Whilst the use of a biotin-streptavidin system was a feasible option, it was not chosen for three reasons (68):

- the FITC/anti-FITC system has been shown to have a similar sensitivity to the biotin-streptavidin system.

- labelling of antibodies with FITC is preferable to labelling with biotin. This is because eluted fractions containing the FITC conjugate are highly coloured which enables easy detection of the success or failure of the conjugation process. In contrast the colourless protein fractions obtained after the biotinylation reaction must be tested further to demonstrate the presence of a conjugate. The fluorochrome content of a FITC conjugate can be determined simply by taking optical density measurements at two wavelengths (280nm and 495nm).
- Studies show enzyme conjugated monoclonal anti-FITC antibody displays extremely low non-specific binding (145). This enables the use of a high concentration of labelled reagents and may contribute to the high sensitivity of assays that employ the FITC/anti-FITC system.

The titration of RV haemagglutinin showed that the size of signal generated was directly proportional to RV haemagglutinin concentration. The higher the concentration of RV haemagglutinin the stronger the resulting signal, most likely due to the greater probability of an RV haemagglutinin molecule coming into contact and binding with captured anti-RV IgG on the solid phase with increasing RV haemagglutinin concentration. As the availability of this reagent was limited, however, a compromise was made and the RV haemagglutinin used at a dilution of 1:10.

To determine optimal concentration of FITC conjugated RV MAB a titration was performed using positive and negative serum controls in both the presence and absence of RV haemagglutinin. The purpose of this experiment was to determine whether the FITC conjugated RV MAB bound non-specifically either to anti-human

IgG bound to the solid phase or to captured RV-IgG. Identical signals were obtained at all FITC conjugated RV MAB dilutions for both the negative control serum in the presence or absence of RV haemagglutinin and the positive control serum in the absence of RV haemagglutinin. The signals were sufficiently low to demonstrate that the FITC conjugated RV MAB did not bind non-specifically to other assay reagents. In contrast, signals obtained using the positive control serum in the presence of RV haemagglutinin were high and began to fall significantly only when the FITC conjugated RV MAB was diluted in excess of 1:3000. Therefore a dilution of 1:2000 of the FITC conjugated RV MAB was chosen, just before the titration curve began to fall away.

A titration of the anti-FITC HRPO conjugate enabled an optimal working dilution to be determined (Fig.9). As for the optimisation of FITC conjugated RV MAB, the experiment was designed to investigate non-specific binding to other assay reagents, particularly to the RV haemagglutinin. The titration curve of the anti-FITC HRPO conjugate in the absence of the FITC conjugated RV MAB showed the $OD_{450/620}$ to decrease from 0.38 to 0.02 as it was diluted from 1:300 to 1:100,000. An $OD_{450/620}$ <0.1 was obtained only for dilutions greater than 1:10,000. These data suggested that if used at dilution factors less than 1:10,000 the anti-FITC HRPO conjugate would bind non-specifically to other assay reagents giving an unacceptably high signal. Therefore a dilution factor of 1:18,000 was chosen for the anti-FITC HRPO conjugate, the highest concentration that could be used without causing non-specific binding.

The experiment investigating variation of substrate incubation time showed that as the substrate incubation time increased optical density readings increased for both positive and negative control sera, the rate of increase of signal being greater for the positive control serum compared to that of the negative control serum (Fig.10). After 25 minutes optical density readings for the positive control serum did not appear to increase. The difference between signals for the positive and negative control sera was greatest after 25 minutes indicating this to be the substrate incubation time giving maximum dynamic range for the assay.

In order to maximise assay sensitivity, incubating each stage of the assay stationary or shaking on a plate shaker at 37°C was investigated (Table 16). The results show that for the positive control serum and all dilutions of the WHO 80 IU/ml standard, with the exception of the 0.625 IU/ml dilution and for the negative control serum, $OD_{450/620}$ values were higher when the microtitre plate was incubated shaking compared to incubating the plate stationary. This difference was shown to be significant by analysis of the logarithm of the $OD_{450/620}$ values by paired t-test ($p < 0.05$). Incubating the plate on a plate shaker therefore gave a wider dynamic range to the assay and allowed a better discrimination between positive and negative results.

A reason for improved assay performance with incubation on a plate shaker is that it is important to maximise contact between reagents already bound to the solid phase and those in solution in order to get the highest positive signals. This can be achieved in three ways. Either increasing the incubation temperature which increases the kinetic energy imparted to each reagent molecule increasing the chances of complimentary reagents meeting, increasing the surface area of the solid phase, or by shaking the

microtitre plate. Since many of the reagents used are temperature sensitive the incubation temperature used was optimised at 37^{0}C which could not be exceeded without risking damage to certain reagents. This temperature also represents "physiological conditions" at which immunological reactions are optimal. As the surface area of the wells of the microtitre plates could not be increased the objective of maximising the chance of immunological reactions taking place between reagents in solution and those bound to the solid phase in the assay was achieved by shaking the microtitre plate on a plate shaker.

Assay evaluation

The performance of the amplified GACELISA was compared to that of the previously described GACRIA (144). The performance of both capture assays for oral fluid testing was also assessed by examining matching serum samples using a sensitive indirect ELISA (Behring) capable of detecting as little as 4 IU/ml of RV IgG. By statistical analysis (kappa and rank correlation) for most of the individual serum/oral fluid panels and overall, results from the oral fluid amplified GACELISA had a higher level of agreement with the results of Behring ELISA on corresponding sera than oral fluid GACRIA (Table 17). In addition, the overall sensitivity and specificity of the oral fluid amplified GACELISA relative to the Behring serum assay was higher than that of the GACRIA (Table 17).

With serum/oral fluid panels 1,2 and 3b (subjects $\leq$ 10 years) results from the amplified GACELISA compared favorably to the Behring serum ELISA with a sensitivity of 94.4%, specificity and positive predictive value of 100% and a negative predictive value of 85% and were similar to those obtained using GACRIA. By

contrast, with panel 3a (an Indian population, subjects ≥ 17 years) results from the amplified GACELISA showed significantly better agreement with the serum Behring ELISA than those from the GACRIA. Also, though both oral fluid assays showed a low sensitivity compared to the serum assay with this panel, the sensitivity of the amplified GACELISA (60.8%) was considerably higher than that of the GACRIA (29.4%) (Table 17).

The reason for the low sensitivity of oral fluid assays with samples from panel 3a may be due to the older age of the subjects providing samples for this panel and the type of assay format used. Reactivity in antibody capture assays depends on the proportion of antibody specific for the antigen under test. The proportion of IgG specific for RV may decrease with age as a consequence of an increase in exposure to other antigens, so may explain the lower sensitivity of capture assays for RV IgG in older subjects. This is supported by the finding that the majority of paired samples giving discordant (serum positive/oral fluid negative) results were from adults and of these the RV IgG GMT of sera was significantly lower than the RV IgG GMT of sera from paired samples giving concordant positive results.

Similar findings of the sensitivity of oral fluid RV IgG decreasing with age was made by Nokes et al (129) using samples from urban and rural Ethiopian communities. There is a need for further investigation of the factors affecting the performance of oral fluid capture assays, particularly in respect to the lack of sensitivity in older age groups. Since in this study the only oral fluid samples representative of an adult population come from India, this issue may be addressed by further age stratified studies using samples from both western and third world populations and could

incorporate the detection of oral fluid IgG to viral antigens other than RV. More basic investigations into the constituents of oral fluid and their affect on the performance of virus specific antibody assays are also required. For example, a further important consideration may be the local production of IgG in oral fluid. Cutts et al (41) suggest that increased local production of IgG in oral fluid may reduce the proportion of total antibody that is specific and therefore lead to a decrease in reactivity in capture assays.

The sensitivity, specificity and predictive values for amplified RV GACELISA (Table 17) have important implications for its use. In adult populations the sensitivity and NPV of amplified GACELISA were low (though higher than those of GACRIA) and there is thus a high probability of false negative results. This may compromise the accurate identification of, for example, immunity in women of childbearing age which is of prime importance when screening adult populations. For pediatric populations (< 14 years), however, the sensitivity and predictive values of the amplified GACELISA for oral fluid samples closely match those of the sensitive Behring serum ELISA. The majority of susceptibles are found in this age group which represent the primary transmission group for RV. The results suggest therefore that the amplified GACELISA could be reliably used for the screening of children for immunity to RV. The amplified GACELISA has now been successfully introduced for routine use with oral fluid samples from the UK rubella surveillance programme (152) where a high proportion of oral fluid samples examined (92% in 1997; Dr M Ramsay, personal communication) are from children under 14 years.

RV IgG prevalence determined by testing oral fluid samples from a rural Ethiopian population

The amplified RV GACELISA was used to screen 831 oral fluid samples for RV specific IgG. All samples were collected using the Oracol device from subjects aged between 0 and 84 years living in the rural district of Butajira in southern Ethiopia (Fig. 3). A matching serum sample was obtained with each oral fluid and was screened for RV specific IgG by Behring ELISA. The purpose of this part of the study was to investigate the epidemiology of rubella in a rural Ethiopian population using oral fluid samples, and to assess the performance of the amplified RV GACELISA as an epidemiological tool.

The highest rate of aquisition of IgG antibody to rubella occurred in the 5-9 year old age group (Fig. 12, Table 19). The majority of persons aged between 16 and 40 years showed evidence of prior infection with RV suggesting that few women of childbearing age are at risk from infection with rubella. The epidemiology of rubella in an urban Ethiopian population from Addis Ababa described by Nokes et al (129) showed a similar trend but there was a lower prevalence for persons aged 0-19 years from the rural population. The highest rate of acquisition of rubella specific IgG antibody from the urban population occurred in those aged 0-4 years. This difference is not unexpected since rubella is an infection transmitted by the respiratory route and so is more easily acquired at an earlier age by those living in an urban environment where the concentration of population is more dense and contact of susceptibles with infected individuals is more likely to occur.

An analysis of results relative to Behring ELISA using serum samples showed the amplified RV GACELISA had an overall sensitivity and specificity of 79% and 90% respectively. The performance of the amplified RV GACELISA closely approached that of Behring for the pediatric population. The sensitivity of the amplified RV GACELISA decreased with increasing age of subjects and assay specificity did not vary with age. These results accurately reflect the perfromance characteristics observed using the age stratified serum-oral fluid panel used to evaluate the assay and support the use of the amplified RV GACELISA as an epidemiological tool, particularly for the screening of pediatric populations which contain the majority of susceptible individuals.

The distribution of RV IgG GACELISA results for oral fluid samples (Fig. 14) from the rural Ethiopian population is consistent with the evaluation of the assay in the comparison with the Behring serum ELISA. The oral fluid assay performed well in comparison to serum ELISA using samples from paediatric populations. This is reflected in the distribution of results for subjects from the Ethiopian population aged <20 years which showed a clear distinction between those positive and negative for RV IgG. In contrast, the distribution of results for those aged >20 years from the Ethiopian population is consistent with the poor sensitivity of the GACELISA in comparison to Behring using samples from older subjects. Results from the serum assay indicates that the majority (>95%) of subjects from the rural Ethiopian population aged >20 years are positive for RV IgG. Therefore the distribution of oral fluid results for these samples should, in theory, lie well to the right of the assay cut-off. That, in practise, these results span the cut-off indicates the poor sensitivity of the RV GACELISA using samples from older subjects. The overall performance of the

RV GACELISA is also illustrated by the scatter graph (Fig. 14) where serum result is plotted against matching oral fluid result for those samples collected from the rural Ethiopian population. Results that fall in the bottom right section of the graph represent false negative results generated by the GACELISA.

In summary, this assessment of amplified RV GACELISA showed it's performance to be superior to that of the previously described GACRIA, with the advantage of a substantially shorter running time in addition to all the benefits of a non-radioactive assay. Although the oral fluid amplified RV GACELISA, like the GACRIA, was not as sensitive as Behring ELISA for serum RV IgG particularly when testing samples from adults, a sensitivity approaching that of the sensitive Behring serum test was achieved when testing oral fluid from children. Moreover, as the amplified GACELISA relative to serum ELISA correlated better and was overall more sensitive than the RV GACRIA, it is a candidate assay for wider use in oral fluid testing.

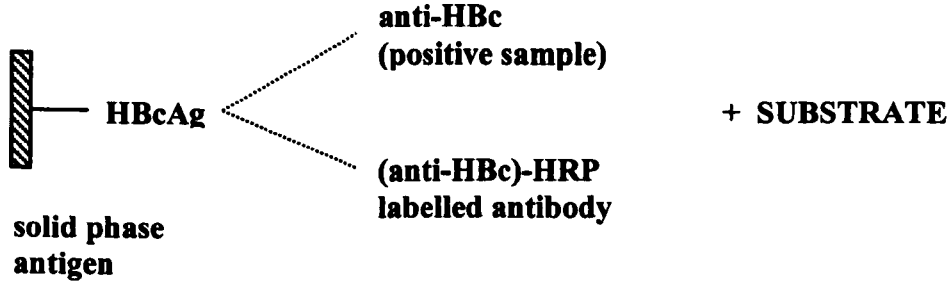
Anti-HBc GACELISA

The anti-HBc GACELISA was developed using experience gained when developing the amplified RV GACELISA. There were, however, four constraints in developing the anti-HBc GACELISA:

1. There was insufficient mouse anti-HBc monoclonal antibody available to develop an assay using the FITC/anti-FITC amplification system as had been done with the amplified RV GACELISA. The minimum practical amount of monoclonal antibody required for conjugation with FITC is 6mg. Therefore assay development was pursued using a detection system comprising the mouse anti-HBc monoclonal antibody and an anti-mouse HRPO conjugate, a format shown to be less sensitive than the amplified ELISA for the RV IgG assay.
2. During initial development of the assay there were no paired serum-oral fluid samples available where the anti-HBc antibody status of the serum had previously been determined, and only relatively few sera were available which were known to be anti-HBc IgG positive. This presented difficulties when trying to evaluate the performance of the assay, particularly when using oral fluid samples. An attempt to overcome this problem was made by "simulating" oral fluid samples by diluting sera by a factor of 1000 so producing a sample with a comparable concentation of IgG to that found in undiluted oral fluid. Subsequently, the evaluation of the assay was carried out using the panel of paired serum-oral fluid samples available from subjects living in the Butajira region of southern Ethiopia.
3. When evaluating the amplified RV GACELISA the WHO 80-IU/ml standard was used when determining assay sensitivity. No comparable standard exists for anti-HBc so a quantitative estimate of assay sensitivity could not be determined.

4. There is no universally accepted gold standard assay for anti-HBc serology. The Hepanostika serum ELISA (Organon) used as a gold standard here does not give quantitative results, unlike the Behring ELISA which was used for evaluating the amplified RV GACELISA. Therefore since the manufacturers do not indicate that results may be considered quantitatively and the Hepanostika serum ELISA is an assay based on a one-step competative inhibition principle (Fig. 24), quantitative comparison between Hepanostika and anti-HBc GACELISA (which uses an antibody capture format) results was viewed with caution.

Figure 24: Hepanostika ELISA assay format (one-step competative inhibition principle)



Assay development and evaluation

Reagent concentrations were optimised in a similar manner to that for the amplified RV GACELISA. An initial titration of positive and negative control sera (Fig. 15) showed that the optical density for the positive control serum decreased with increasing dilution, with a strong signal still obtained at a dilution of 1:100,000. In contrast, the optical density for the negative control serum remained at a constant value of <0.1 until a dilution factor in excess of 1:30,000 was reached when the signal began to rise. Therefore sera were tested at a dilution of 1:1000 and oral fluid tested undiluted.

These observations suggested that when using samples that contained a particularly low antibody concentration, as can be the case with oral fluid samples, the potential exists for certain reagents to bind non-specifically to other assay reagents. Threfore each reagent used during each stage of the assay was assessed for its potential to bind non-specifically to previously added reagents and to therefore cause false positive reactions. Experiments showed that the only reagent which bound non-specifically was the anti-HBc monoclonal antibody, which bound to any unsaturated rabbit anti-human IgG on the solid phase (Table 20). This phenomenon had previously been encountered by Vyse et al (186) when developing an assay for detecting oral fluid IgG antibody to Epstein Barr virus and was countered by including antibody negative human sera (NHS) in the monoclonal antibody diluent. This saturated any available rabbit anti-human IgG with antibody other than that specific for the virus that was the target of the assay, so preventing the non-specific binding of the monoclonal antibody without adversely affecting the resulting signal. Therefore 5%NHS was included in

the anti-HBc monoclonal antibody diluent and subsequent experiments showed that this effectively prevented any non-specific binding (Table 21).

A titration of the rHBcAg against the positive control serum (Fig. 16) provided information on the performance characteristics of the assay as well as assisting in determining optimal rHBcAg concentration. The size of signal generated decreased with increasing dilution of the positive control serum but only significantly so once the dilution of the positive control serum had exceeded 1:30,000. These observations suggested that the assay was sufficiently sensitive to detect the levels of virus specific IgG expected in oral fluid. Further support that the anti-HBc GACELISA was sufficiently sensitive for use with oral fluid samples was lent by the titration of oral fluid samples obtained from two persons confirmed as anti-HBc antibody positive. When tested undiluted both oral fluid samples gave a strong signal which decreased as the oral fluid samples were diluted and signals were still strong enough to be considered as positive at an oral fluid dilution of 1:32.

A preliminary evaluation suggested that, relative to the Hepanostika ELISA, the anti-HBc GACELISA lacked sensitivity but was specific (Table 22). That 5 of 6 sera testing positive by anti-HBc GACELISA still gave a positive result at high sample dilutions indicated that the performance of the anti-HBc GACELISA may be dependant upon the proportion of HBc IgG present in samples and the assay may only be useful for screening those oral fluid samples containing a high proportion of anti-HBc specific IgG. It should also be noted that the Hepanostika ELISA measures ***total*** HBc antibody whilst the GACELISA is anti-HBc IgG specific. However, in the panel screened, the majority of HBc antibody present was likely to be of the IgG class since

they were not known to be from cases of recent, acute or chronic hepatitis B. Further conclusions concerning the performance of the anti-HBc GACELISA with oral fluid samples could not be made until the assay had been evaluated using a suitable panel of paired serum-oral fluid samples.

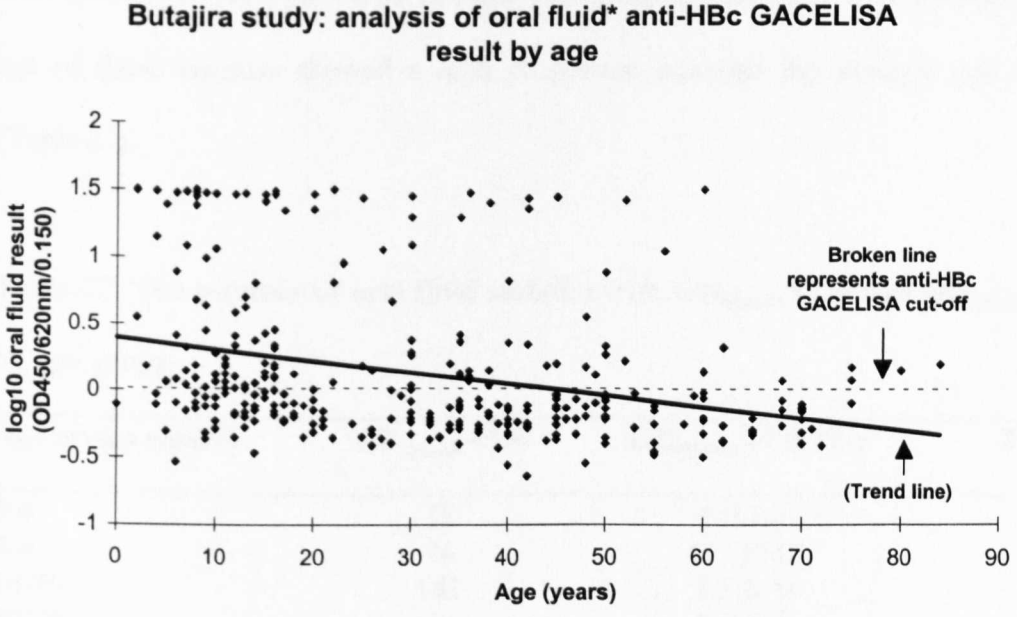
An evaluation was performed using serum-oral fluid samples from Butajira. Of the 831 matched samples used for the rubella study previously described, 538 had sufficient volume remaining to be screened for anti-HBc antibody. Sera were screened using the Hepanostika ELISA (Organon) and the matching oral fluids screened using the anti-HBc GACELISA. The use of this panel allowed not only an assessment of the oral fluid anti-HBc GACELISA, but also permitted some analysis of the epidemiology of HBV infection in a rural Ethiopian population.

The results from serum samples screened by Hepanostika ELISA showed that the prevalence of anti-HBc increased with age from 22% in the group aged 0-4 years to 80% in those aged over 30 years (Fig. 20). These results illustrate the high prevalence of HBV infection in African communities where approximately 8% are chronically infected (100).

Relative to Hepanostika ELISA on serum, the anti-HBc GACELISA on corresponding oral fluid samples (from Butajira) had an overall sensitivity and specificity of 43% and 87% respectively, with a positive predictive value of 83% and a negative predictive value of 50% (Table 24). An analysis of these results by age group (Table 26) showed that, as for the amplified RV GACELISA, the sensitivity of the anti-HBc GACELISA decreased with age. This is supported by the analysis shown

in Fig.25, a scatter graph plotting age of subject against the logarithm of anti-HBc GACELISA result from oral fluid samples where the matching serum sample tested positive for anti-HBc by Hepanostika ELISA. This shows the proportion of positive results, particularly those in the upper limit of the dynamic range of the assay, to decrease with age. Reasons for the decrease in sensitivity of the anti-HBc GACELISA with increasing age of subject may be similar to those previously discussed for the amplified RV GACELISA. However, the overall sensitivity of the anti-HBc GACELISA was considerably lower than that of the amplified RV GACELISA, even for samples from pediatric age groups.

Figure 25



*oral fluids from subjects where matching serum was anti-HBc Hepanostika positive

The dynamic range of the anti-HBc GACELISA observed using the Butajira oral fluid samples was wide, with $OD_{450/620}$ values ranging from 0.034 to 4.767, and was considerably greater than that observed for the amplified RV GACELISA. The anti-

HBc GACELISA, as with the amplified RV GACELISA and the majority of other oral fluid assays described in the literature, was developed using an antibody capture format and so measures the proportion of anti-HBc IgG antibody present in a sample relative to the total IgG antibody present. The broad dynamic range seen when screening the oral fluid samples from Butajira suggests that the anti-HBc IgG antibody response varies considerably in individuals within this population, with some individuals showing a particularly high proportion of total IgG to be anti-HBc specific (100).

Of the 538 oral fluid samples from the Butajira study that were screened by anti-HBc GACELISA, 48 (9%) gave a particularly strong signal ($OD_{450/620}$ >1.0). An analysis by age of these samples showed a high proportion amongst the younger age classes (Table 27).

Table 27. The number of oral fluid samples with $OD_{450/620}$ <1.0 and $OD_{450/620}$ >1.0 by age group

Age group (years)	$OD_{450/620}$ <1.0	$OD_{450/620}$ >1.0 (%)	TOTAL
0-4	38	6 (13.6)	44
5-9	74	12 (14.0)	86
10-19	143	10 (6.5)	153
20-29	40	7 (14.9)	47
30-49	115	9 (7.3)	124
50+	74	4 (5.1)	78
TOTAL	484	48 (9.0)	532

It is possible that samples with $OD_{450/620}$ >1.0 came from persons recently infected with HBV where the proportion of specific anti-HBc would be expected to be high.

Further serology measuring HBsAg and anti-HBs would help to determine the status of HBV infection in these cases but financial constraints and an insufficient volume of serum remaining prevented this work being done.

The distribution of anti-HBc IgG GACELISA results for oral fluid samples from the rural Ethiopian population (Fig. 21) is consistent with the low sensitivity of the assay compared to that of the Hepanostika ELISA that was used for matching serum samples. Overall, two distinct populations of results were found, one corresponding to negative samples and another to strongly positive samples and this did not vary by age group. Results using the Hepanostika ELISA on matching sera, which were considered as the 'true' result for the purpose of this study, showed the prevalence of anti-HBc antibody to increase with age from 20.5% in those aged 0-4 years to 78.2% in those aged 50+ years (Table 25). Therefore this should be reflected in the age stratified distribution of oral fluid results in Fig. 21. That no difference in the distribution for corresponding oral fluid results was seen between the age groups highlights the decrease in sensitivity with increasing age of subjects and demonstrates that the assay performs most reliably in the younger age classes.

Further evidence of the relatively poor performance of the anti-HBc GACELISA in comparison to Hepanostika is given by the scatter graph in Fig. 22 where GACELISA results ($\log[OD_{450/620}/0.150]$) were compared to corresponding serum results (% inhibition). Whilst the manufacturers do not recommend such a quantitative expression of results when using the Hepanostika ELISA, it does serve to illustrate how individual results by each of the assays compare. The large proportion of results which cluster in the bottom right hand section of the graph represent false negative

results generated by the anti-HBc GACELISA whilst those in the top right hand section represent false positives, demonstrating a lack of sensitivity and specificity. The distribution of results with respect to the oral fluid assay also highlights the difficulty in choosing a cut-off in contrast to that for the serum assay, where a clear distinction can easily be made.

When evaluating the performance of the anti-HBc GACELISA with oral fluids important considerations are the characteristics and format of the serum assay against which the oral fluid assay is being compared. Assays that use a competitive principle, such as Hepanostika anti-HBc Uni-Form ELISA, have been shown to give a clearer distinction between positive and negative samples (197). They therefore tend to be more specific than other assay formats commonly used in ELISAs, such as the indirect method. Since competitive assays are highly specific (139), they enable test serum to be used undiluted or at a low dilution which enhances sensitivity with samples containing only low levels of the antibody. The use of a competitive assay for detecting anti-HBc in oral fluid samples was considered not suitable, since the expected levels of anti-HBc would be considerably lower than that found in serum and so would not be present in high enough concentrations to compete with the anti-HBc conjugate.

Therefore, when evaluating the performance of the anti-HBc GACELISA with oral fluid samples relative to that of Hepanostika with serum samples, the following points should be taken into consideration:

- the Hepanostika assay measures total anti-HBc antibody whilst the anti-HBc GACELISA measures only HBc specific IgG.

- the competitive format used by Hepanostika, together with the use of undiluted test serum, provides a level of sensitivity able to detect low levels of anti-HBc, such as those present in a sample representing infection in the distant past. The anti-HBc GACELISA was developed using an antibody capture principle, a format thought to be that most appropriate for use with oral fluid samples, but which is not necesssarily the most sensitive of assay formats for other sample types.
- There is no universally recognised "gold standard" assay for detecting anti-HBc IgG.

An interesting follow up experiment would be to take serum samples which were positive by Hepanostika but the corresponding oral fluid negative by GACELISA, and rescreen the serum in the Hepanostika ELISA at a dilution factor of 1000 so the levels of anti-HBc are equivalent to those found in undiluted oral fluid. This would help to determine the limit of sensitivity of antibody capture assays with reference to the level of specific antibody in serum. It may be that the level of antibody in individuals with past infection can be detected in serum using suitable sensitive assays but cannot be detected in oral fluid using the current technology available.

That the sensitivity of the anti-HBc GACELISA decreases with age of subject and that the majoritory of oral fluid samples highly reactive were provided by persons in the younger age classes is consistent with the the hypothesis that after infection with HBV the initial anti-HBc response is very strong but decreases with time to a level not always detectable in oral fluid by capture assays. Only by the use of sensitive competitive assays where serum is added undiluted can anti-HBc be detected.

The performance of the anti-HBc GACELISA with 334 oral fluid samples collected by salivette device was compared to that of the Murex ICE test which also uses a capture format but detects total anti-HBc and can be adapted for use with oral fluid samples. The results (Table 24) suggested there was little difference in performance between the modified ICE and GACELISA tests. Moreover, it was not possible to resolve discrepancies by testing with anti-HBc GACRIA, which has now been superseded by the Murex ICE test as the method of choice for screening oral fluid samples for anti-HBc in the Hepatitis and Retrovirus Laboratory, Central Public Health Laboratory.

An interesting comparison between the performance of the Murex ICE test and anti-HBc GACELISA with oral fluid samples can be made using the results of a study by Nigatu et al (unpublished data 1996). Nigatu and colleagues collected 423 paired serum and oral fluid samples from an urban population in Addis Ababa, Ethiopia. The sera were screened for total anti-HBc using the Hepanostika ELISA and the matching oral fluids tested for total anti-HBc using the Murex ICE ELISA. Analysis showed the ICE ELISA to have an overall sensitivity and specificity of 36% and 92% respectively compared to Hepanostika. The poor performance of ICE with these African samples highlighted the need for an oral fluid assay with suitable performance charateristics for use in developing countries where prevalence of HBV infection is high. The results with the ICE ELISA were similar to those of the anti-HBc GACELISA relative to Hepanostika (using samples from Butajira). The anti-HBc GACELISA had a slightly higher sensitivity but lower specificity than ICE. However, unlike the anti-HBc GACELISA, analysis of the ICE ELISA results did not show an age related sensitivity effect. Therefore the performance of the anti-HBc GACELISA may be

considered a minor improvement over ICE when screening oral fluid samples from younger subjects from African populations.

The Murex ICE ELISA, however, has been shown to perform well using oral fluid samples collected from intravenous drug users (IDUs) from Western European populations. It has a high level of sensitivity and specificity relative to matching serum samples tested by serum ELISA (JV Parry, personal communication 1999), and is currently the assay used for screening oral fluid samples for anti-HBc in the Hepatitis and Retrovirus Laboratory, CPHL. The poorer assay performance seen when using African samples may be related to the type of population used for the analysis. Samples from IDU populations are likely to represent recent HBV infection where the proportion of anti-HBc is high. In contrast, the African study population also contains persons with distant infection who thus have a much lower proportion of specific antibody.

For epidemiological screening an oral fluid assay ideally requires a level of sensitivity to be at least 90% relative to serum and a correction factor used to compensate for any underestimate of prevalence. As the anti-HBc GACELISA currently stands it has only limited used for epidemiological screening, even with the use of correction factors, due to the low level of sensitivity relative to that of the reference serum assay. Unlike the amplified RV ELISA, this was the case even for samples from pediatric groups where performance of the assay was at its best. The assay appeared to perform satisfactorily using oral fluid samples that contained a particularly high proportion of anti-HBc antibody, possibly from persons with a recent HBV infection or those who

may be carriers. The use of an FITC/anti-FITC amplification system could be investigated to improve the level of sensitivity of the anti-HBc GACELISA.

This work suggests that a successful anti-HBc ELISA that can be used with oral fluid samples with a performance approaching that of recognised serum assays will be difficult to achieve with the current ELISA technology available. Therefore further work should focus upon the development of new and more sensitive detection systems capable of producing a suitable signal even with only very low levels of the antibody. It will be imperative that such systems have a high specificity, ideally 100%, to avoid amplifying false positive reactions. It may be possible to pursue such a detection system by coupling conventional ELISA techniques with polymerase chain reaction (PCR) technology, producing an immuno-PCR. Such a technique has been described (166) and made use of a streptavidin-protein A chimera which has a very tight and specific binding affinity for biotin and IgG. This enables biotinylated DNA to be attached to a MAB. The ELISA can therefore be carried out in a conventional manner but, rather than use a detection system comprising an HRPO conjugated antibody and a chromogenic substrate to produce a colourimetric signal, a monoclonal antibody was used to which a piece of DNA is attached designed to contain specific primer sites which are detected by a PCR reaction. Nested PCR reactions can be made sensitive enough to detect a single copy of the target. In theory it should therefore be possible for such a system to detect a single target antibody bound to the solid phase, though it may not be as suitable for use in the field as a conventional ELISA for practical reasons. In comparison to conventional ELISA, immuno-PCRs have been demonstrated to improve sensitivity by a factor of 10^5 (123, 166). However, the

technique has mostly been applied to the detection of antigen and has yet to be fully investigated for antibody detection.

ROC analysis of RV GACELISA and anti-HBc GACELISA

ROC analysis (23) can be used to determine an appropriate cut-off for an assay. It requires a population of samples to have been screened by both the assay under consideration and by a gold standard assay so a "true" result is available. ROC analysis is particularly useful for comparing assays. Mixture modelling (137) is an alternative method which can also be used to set an assay cut-off and has the advantage of not requiring a "true" result and is particularly useful for setting cut-off values for screening assays. It was not used in this study as "true" results were available for the samples screened.

The ROC curves generated for the two assays developed in this study showed that neither performed close to "perfect" in comparison to the gold standard result determined by commercial ELISA on matching serum samples as neither came close to passing through the ideal point (Fig. 23). Of the two assays the RV GACELISA performed better in this respect than the anti-HBc GACELISA. This observation reflects the assessments made of the performance characteristics of each assay by other criteria.

For both assays the flatness of the ROC curve around that region of the curve with a gradient of 1.0 made it difficult to identify the precise location of the point that represented the cut-off which gave the highest sensitivity and specificity with respect to the gold standard results. Therefore an approximate region for this point was

indicated (Fig. 23). That the cut-off chosen for the RV GACELISA during assay development fell within this region suggests that this cut-off was satisfactory for use of the RV GACELISA for determining population immunity. In contrast, the cut-off chosen for the anti-HBc GACELISA did not fall close to the point which represented the highest sensitivity with minimum effect on specificity, and erred on the side of a higher specificity at the expense of sensitivity. This cut-off is therefore more appropriate for an assay designed to be used for individual diagnosis rather than for screening for population immunity. The ROC curve suggested that a cut-off $OD_{450/620}$ of 0.1 (rather than 0.150) would provide the assay with highest combined sensitivity (66.7%) and specificity (66.4%) for screening purposes.

This ROC analysis highlighted some of the pitfalls associated with choosing an assay cut-off and demonstrated that the intended use of the assay is a prime consideration influencing the approach taken when defining the cut-off. Techniques such as mixture modelling or ROC analysis should be used to set cut-off values for assays intended for diagnosis of immunity in the population as these techniques consider assay performance using a population of interest and enable a cut-off to be set that ensures maximum sensitivity and specificity. This means that the cut-off may have to be set ***after*** the assay has been optimised and used for its intended purpose on a population of study samples. In contrast the cut-off for assays designed for diagnosis in the individual should be investigated using a carefully selected panel of well evaluated samples where the true status is known as determined by a gold standard assay, and the cut-off set to err on the side of higher specificity at the expense of sensitivity ***before*** the assay is used. In such a situation it may therefore be appropriate to use the signal from a population of negative samples and apply the mean plus two or three

standard deviations as a method for setting the cut-off, particularly if the estimate for the standard deviation is high.

Factors affecting the performance of oral fluid assays

The final part of this discussion focuses on the different performance characteristics seen for the oral fluid assays developed both in this study and with others described in the scientific literature, and considers the factors which may affect and influence assay performance.

These investigations have illustrated the suitability of oral-fluid collection for large scale epidemiological investigation of antibody prevalence to virus infections in rural communities in developing countries. They have also highlighted the uses and limitations of conventional ELISA systems with oral fluid samples which contain only low levels of the specific antibody of interest compared to serum. Assay sensitivity for both the amplified RV GACELISA and the anti-HBc GACELISA decreased with time elapsed since original infection, assuming this to be proportional to age. Whilst the agreement between serum and oral fluid was close for those aged <20 years for rubella, agreement between serum and oral-fluid was poor across all age groups using the anti-HBc GACELISA.

Subsequently, a GACELISA for the detection of measles specific IgG in oral fluid has been developed (126). This was based on the FITC/anti-FITC amplification system and was used to screen the same population of oral fluid samples from rural Ethiopia as for the amplified RV GACELISA and anti-HBc GACELISA. In contrast to the rubella and HBV assays, the agreement between serum and oral fluid for measles

specific IgG antibody was close for all ages, the overall sensitivity and specificity being 97% and 93% respectively. Analysis showed that whilst measles antibody levels were shown to decline with age for both serum and oral fluid, this did not occur to such an extent as to effect the sensitivity of the oral fluid assay in contrast to those for rubella and anti-HBc. A comparison of all three oral fluid assays therefore shows considerable variation in performance characteristics, the measles assay performing best followed by the amplified RV GACELISA and lastly the anti-HBc GACELISA.

The different performance characteristics seen for the three oral fluid assays may simply be a reflection of the quality of reagents, in particular that of the antigen used. However, an alternative explanation for the superior assay performance characteristics observed for the measles GACELISA with oral fluid may be provided by a study of nonclassic measles infections in an immune population exposed to a single index case made by Helfand et al (70). The conclusions of this study are that mild/asymptomatic measles infections are probably common, and may be the most common manifestation of measles during outbreaks in highly immune populations. Infection with measles virus under such circumstances has the effect of boosting the immune system and maintaining a high proportion of neutralzing antibody. Therefore a measles antibody capture assay designed for use with oral fluid samples may be expected to perform well across the age spectrum, particularly when screening populations from developing countries, such as those in rural Ethiopia, where there is a high level of natural immunity, poor vaccination coverage, and regular epidemics of measles.

This leads to the hypothesis that the titre of neutralising antibody and performance of an oral fluid assay may be related to the frequency which the immune system

encounters the antigen in question. For viruses transmitted by the respiratory route, such as measles and rubella, this might be estimated by measuring the basic reproduction number (R_0) which is defined as the number of secondary cases of infection generated by one primary infected host when introduced into a totally susceptible population (2, 160). Infections with a high R_0 (and where a suitable number of index cases occur within the population) are more likely to cause mild/asymptomatic infections during outbreaks in highly immune populations. It can be predicted that oral fluid capture assays for these virus infections would perform well across the age spectrum. This hypothesis is supported by a comparison of the performance characteristics for the oral fluid capture assays developed for measles, rubella and anti-HBc and estimates of R_0 made for each of these virus infections for the rural Ethiopian population studied (2, 192). Estimates for R_0 in an African population show that R_0 for measles is > R_0 for rubella which is > R_0 for HBV.

In addition, oral fluid assays for viruses causing persistent infection may perform well across the age spectrum because the immune system encounters the virus every time it reactivates from its latent state. This idea was supported by the development of a successful oral fluid capture assay for IgG specific antibody to the viral capsid antigen of EBV (186). For similar reasons oral fluid capture assays for specific IgG antibody to antigens of HIV perform well. However, because of the high death rate following HIV infection, the possibilities of observing the performance of such assays using samples obtained 10 years or more after initial infection are limited.

CONCLUSIONS

A major objective of this study was to develop simple, sensitive and robust ELISA-based oral fluid antibody detection methods for rubella and anti-HBc IgG that can be used to replace existing radioimmunoassay technology. They should be suitable for use in both developing and developed countries, though the emphasis has been on producing assays that were particularly amenable for use in basic laboratories in developing countries. Ideally, both assays should be suitable for use in the field. Whilst this objective has not been entirely met, the work has been valuable in demonstrating some of the limitations in current ELISA technology available for producing oral fluid assays with a comparable level of performance to serum ELISA. It has also demonstrated the difficulties there are in setting the most suitable cut-off for a screening assay.

Both the RV GACELISA and the anti-HBc GACELISA were relatively simple to perform and less technically demanding than radioimmunoassay. In this respect both are suitable for use in laboratories in developed countries and may be considered as candidate assays for use in basic laboratories in developing countries. Possible practical problems which may be encountered in transferring the assays to more basic laboratories are the availability of reagents and the lack of equipment such as a plate washer, ELISA plate reader and plate shaker/incubator. Whilst this equipment is considered as standard in most laboratories in the developed world, it is relatively expensive and requires regular maintenance and training to be used effectively. These are requirements that are not often satisfactorily met in the developing world. Many laboratories only possess a limited range of equipment that is often second hand or considered as obsolete by western standards. Appropriate equipment and its

maintenance are required if the RV GACELISA, anti-HBc GACELISA and similar assays are to be used in laboratories in the developing world. If oral fluid assays are to be used under field conditions considerable simplification is required. This will involve making the assay running time significantly shorter (minutes rather than hours) and eliminating the need for more complex laboratory equipment that requires an electrical power supply. Perhaps the ideal oral fluid assay may consist of a "dip stick" type test where appropriate reagents are immobilised on a solid phase which can be dipped into a sample of oral fluid and the qualitative determination of specific antibody made visually by the operator by the presence or absence of colour in a matter of seconds. In this way a result could be provided "on the spot" and any subsequent action needed regarding the administration of an appropriate vaccine acted upon immediately.

Both the RV GACELISA and anti-HBc GACELISA lacked sensitivity compared to serum ELISA, and the sensitivity of both assays was shown to decrease with increasing age of subjects. Whilst the performance of the RV GACELISA was shown to be an improvement over the existing radioimmunoassay (and could act as a replacement assay) and performed particularly well with samples from paediatric populations in comparison to serum ELISA, the performance of the anti-HBc GACELISA was not considered good enough to warrant further use. Despite these results, the subsequent development of a successful measles GACELISA (126) suggests that it is possible to produce useful oral fluid assays using standard techniques that are capable of performing well in comparison to serum ELISA. However, the performance of the RV GACELISA and anti-HBc GACELISA indicate that current limitations in ELISA technology need to be considered in conjunction

with the biological characteristics of a micro-organism and the immune response generated against it, before embarking on the development of further oral fluid assays. This work has highlighted the need to develop a new generation of oral fluid assays with a level of sensitivity high enough to reliably detect the low levels of specific antibody that may be present in oral fluid samples, particularly those from subjects where infection was in the distant past. One approach may be to use immuno-PCR though a disadvantage of this method is that it is unlikely to be suitable for use in basic laboratories.

The need to develop assays suitable for use on oral fluid rather than serum can be related to the importance of an infectious disease, the availability of an effective vaccine and the need to implement and monitor vaccination campaigns. These are factors that will vary between countries and particularly between the developed and developing world. To date almost all oral fluid assays described in the literature have been specific to virus infections, with little research into those for detecting specific antibody to bacterial or important parasitic antigens. The development of oral fluid assays capable of detecting specific antibody against important bacterial infections, particularly those for which a vaccine is available, is therefore an area which could be investigated further. For example, a vaccine against meningococcus C is currently being used in the UK in an attempt to help protect the population against meningococcal infection (149). An oral fluid assay would therefore be useful for the surveillance of meningitis C infection.

APPENDIX 2

2.1 Hepanostika anti-HBc Uni-Form

2.1a Reagent preparation

- Reagents should be at room temperature (15 - 30°C) before beginning the assay and can remain at room temperature during testing.
- The foil pack containing micoelisa strips should be brought to room temperature before opening.
- Check the phosphate buffer concentrate for the presence of salt crystals. If crystals have formed in the solution resolubilize by warming at 37°C until crystals dissolve. Dilute the phosphate buffer concentrate 1:25 with distilled water. Prepare at least 25ml of buffer for each microelisa strip used and mix well before use.
- TMB substrate: combine the required amounts of TMB solution in a new disposable vial in equal parts with urea peroxidase solution according to the number of wells being run (see chart included with kit insert). Mix well. Protect the TMB solution and TMB substrate from excessive light. TMB substrate must be almost colourless when used.
- Sulphuric acid (analytical grade) must be prepared at a concentration of 1 mol/l.

2.1b Test procedure

- Fit the stripplate with the required number of Microelisa strips.
- Pipette 100ul of each test sample into the wells. Pipette the controls after the samples. Include three negative controls (100ul each) and three positive controls (100ul each) in each stripplate.
- Pipette 50ul conjugate into each well with sample or control. Agitate (e.g. using a microshaker, speed approx. 900 rpm) for 15 seconds.

- Incubate at 37°C for 90 minutes.
- Wash: aspirate the wells completely and fill completely with phosphate buffer and allow to soak (30 - 60 seconds). Aspirate completely and repeat the wash and soak procedure three additional times for a total of four washes.
- Pipette 100ul TMB substrate into each well.
- Incubate at 15 - 30°C for 30 minutes.
- Stop the reaction by adding 100ul 1 mol/l sulphuric acid to each well.
- Read plate: blank reader on air (without stripplate) and read the absorbance of the solution in the wells at 450 $\pm$5 nm.

2.1c Results

- Calculations must be made separately for each stripplate (**N** = mean absorbance of the negative controls; **P** = mean absorbance of the positive controls; **S** = mean absorbance of the test sample).
- Elimination of outlying positive control values: before the test results are determined, outliers in the values found for the negative and positive controls must be eliminated:

1. *Eliminate individual negative controls with an absorbance $\leq$ 0.750*
2. *Eliminate individual positive controls with an absorbance $\geq$ 0.300*
3. *Calculate N and P*
4. *Eliminate individual negative controls with an absorbance < 0.7N or > 1.3N*
5. *Recalculate N and repeat 4 if necessary*

- Checking of test-run validity: a test-run is only valid if less than half the number of controls have been eliminated and N - P $\geq$ 0.500
- Calculatio of cut-off value: the cut-off value is 0.25(N+3P)

- Test result: a test is positive if S $\leq$ cut-off value; a test is negative if S > cut-off value.

2.2 ICE HBc Detection Pack

2.2a Preparation

- Conjugate preparation: reconstitute a bottle of Conjugate with a single bottle of Conjugate diluent. Mix well.
- Substrate solution: add a volume of colourless Substrate Diluent to an equal volume of pink Substrate Concentrate (alternatively pour the entire contents of the bottle of Substrate Diluent into the bottle of Substrate Concentrate, which provides sufficient reagent for 5 plates).
- Wash Fluid: dilute the contents of one bottle of Wash Fluid to a final volume of 2500ml with distilled water.
- Stop Solution: prepare 0.5M sulphuric acid (analytical grade).

2.2b Test Procedure

- Reconstitute and mix the conjugates and prepare the substrate solution.
- Use only the number of wells required for the test.
- Add 50ul of sample diluent to each well.
- Add 50ul of samples or controls to the wells. For each plate use the first column of wells for the assay Controls. Add the Controls to the designated wells after dispensing the samples. Pipette 50ul of the Negative Control into each of three wells A1 to C1 and 50ul of Hbc Positive Control into wells D1 and E1.
- Cover the wells with the lid and incubate for 30 minutes at 37^{0}C.
- Wash plate: perform 5 wash cycles using working strength Wash Fluid ensuring that:

1. *The 'fill volume' is 500ul/well*
2. *The 'dispense height' is set to completely fill the well without causing an overflow.*
3. *The time taken to complete one aspirate/wash/soak cycle is approximately 30 seconds.*
4. *Where possible a 'double aspirate' step is used on the final cycle to ensure that no liquid is left in the well.*

- Add 50ul conjugate to each well.
- Cover the wells with the lid and incubate for 60 minutes at 37°C.
- Wash as previously described.
- Add 100ul of substrate solution to each well.
- Cover the wells with the lid and incubate for 30 minutes at 37°C. Keep away from direct sunlight.
- Add 50ul of Stop Solution (0.5M sulphuric acid).
- Read the absorbance at 450 nm using 620 nm to 690 nm as the reference. Blank the instrument on air.

2.2c Quality Control

- Results of an assay are valid if the mean absorbance for the Negative Control is < 0.2, the mean absorbance for the Positive Control is more than 0.8 above the mean absorbance of the Negative Control.

2.2d Results

- Each plate must be considered separately when calculating and interpreting results of the assay.

- Calculate the mean absorbance of the Negative Control. If one of the Negative Control Wells has an absorbance of > 0.15 above the mean of all three discard that value and calculate the new Negative Control mean from the two remaining replicates.
- Cut-off Value: calculate the cut-off Value by adding 0.2 to the mean of the Negative Control replicates.
- Samples giving an absorbance < the Cut-off Value are considered negative.
- Samples giving an absorbance ≥ the Cut-off Value are initially considered reactive in the assay. Such samples should be retested in duplicate using the original source. Samples that are reactive in at least one of the duplicate retests are considered repeatably reactive in the ICE HBc test and are presumed to contain antibodies to HBc. Such samples should be investigated further. Samples that are non-reactive in both wells on retest should be considered non-reactive for HBc antibodies.

CHAPTER 3

THE MOLECULAR EPIDEMIOLOGY OF RUBELLA VIRUS USING RT-PCR, AND THE ROLE OF ORAL FLUID IN THE DETECTION OF VIRUS GENOME

Objectives:

- To develop a reverse transcription polymerase chain reaction (RT-PCR) assay suitable for studying the molecular epidemiology of rubella and assisting with diagnosis.
- To use the RT-PCR assay to study the molecular epidemiology of RV using suitable specimens.
- To investigate the role of oral fluid as a suitable clinical specimen for the detection of measles, mumps and rubella virus genome.

Introduction

To date there have been few studies investigating the molecular epidemiology of rubella, in contrast to other vaccine preventable virus infections such as measles and mumps. The work presented in this section of the study will contribute to the understanding of the extent of RV strain diversity.

This study investigated the geographic and temporal distribution of RV strains that were available using a reverse transcription polymerase chain reaction (RT-PCR) assay designed to target a region of the E1 gene of RV. The E1 gene was chosen because previous studies have shown that humoral responses are directed mainly against the glycoprotein encoded by the E1 gene during RV infection. This therefore indicates that the E1 gene product may be a site of antigenic variation (143) suggesting that it may be interesting for molecular epidemiological studies of RV.

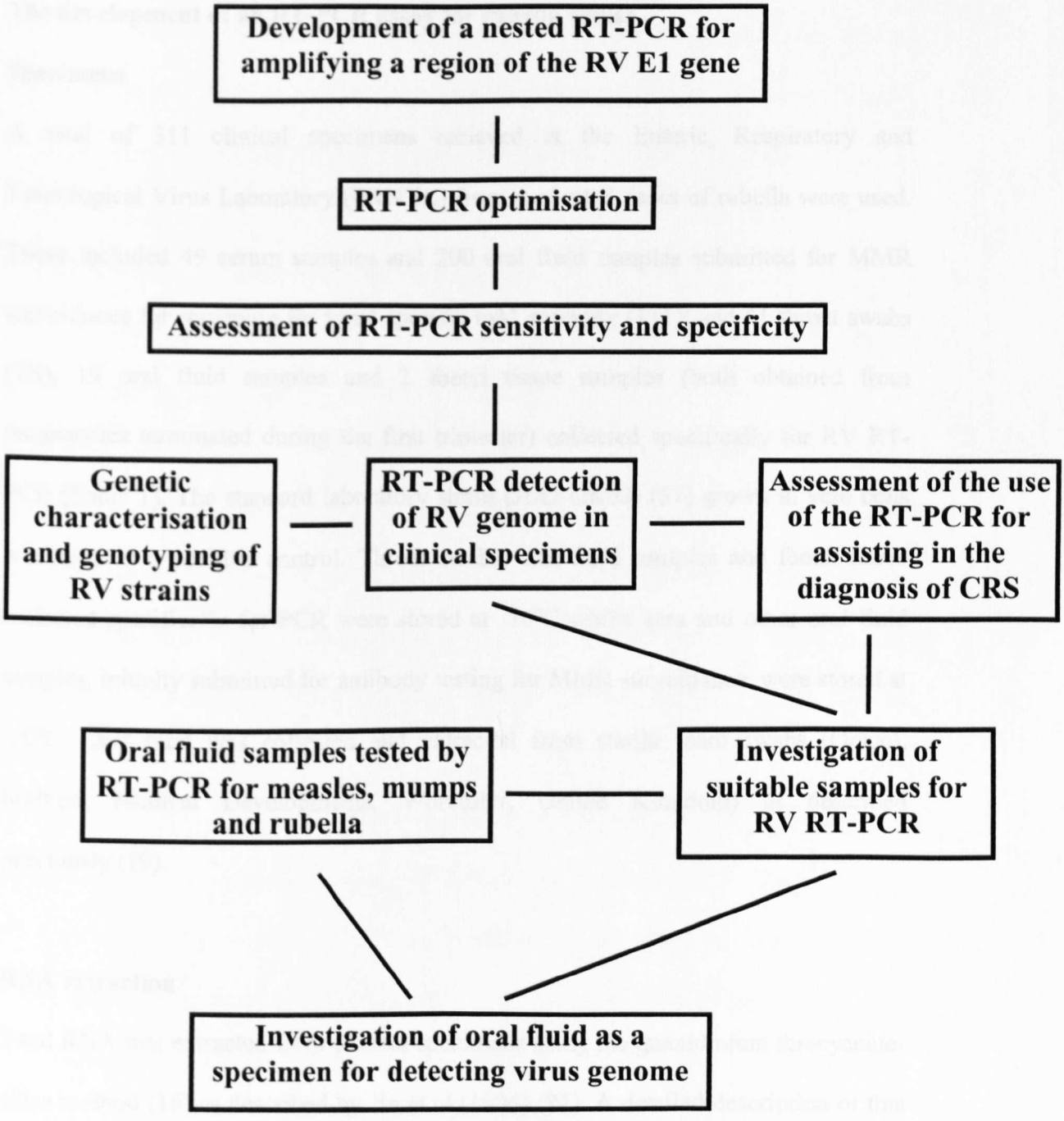
The role of oral fluid samples for the detection of measles, mumps and rubella virus genome was investigated. The extent to which oral fluid may be used in molecular epidemiological studies of infections with these viruses was studied. This is of interest as oral fluid is a clinical specimen with significant compliance advantages over other types of specimen that are currently used which include throat swab, nasphyrangeal aspirate, blood and urine. Measles, mumps and rubella were chosen for this part of the study because they are all important vaccine preventable virus infections that are transmitted by the respiratory route. The close proximity of the oral cavity to the respiratory tract (which is a portal of entry and initial site of replication for respiratory transmitted viruses) suggests that oral fluid is likely to be a suitable specimen for genome detection and subsequent genotyping work for respiratory transmitted viruses.

This does not imply, however, that the use of oral fluid for the detection of virus genome is exclusive to viruses transmitted by the respiratory route. It should also be possible to detect in oral fluid the genome of any acute virus infection that gives rise to a viraemia due to transudation of virus from the blood to the crevicular fluid. Other viral genomes that have been detected in oral fluid by PCR include HIV-1 (98),

human T-lymphotropic virus type 1 (HTLV-1) (1), hepatitis C (HCV) (83), hepatitis G (HGV) (22), transfusion transmitted virus (TTV) (162), rabies (38), human herpes viruses (HHV)-6, HHV-7, HHV-8 (99), Epstein-Barr virus (EBV) (78), cytomegalovirus (CMV) (99) and herpes simplex virus type 1 (HSV-1) (176). Practical aspects such as timing of oral fluid collection and storage of samples prior to PCR analysis were also investigated.

An outline plan of the study is shown in the flow chart (Figure 26).

Figure 26: A plan of the study (chapter 3)



MATERIALS AND METHODS

The development of an RT-PCR assay for rubella virus:

Specimens

A total of 311 clinical specimens recieved at the Enteric, Respiratory and Neurological Virus Laboratory (ERNVL) from suspected cases of rubella were used. These included 49 serum samples and 200 oral fluid samples submitted for MMR surveillance for screening for virus specific IgM antibody (111); and 41 throat swabs (TS), 19 oral fluid samples and 2 foetal tissue samples (both obtained from pregnancies terminated during the first trimester) collected specifically for RV RT-PCR (Table 1). The standard laboratory strain (JUD-UK62) (57) grown in vero cells was used as a positive control. Throat swabs, oral fluid samples and foetal tissue collected specifically for PCR were stored at -70^{0}C whilst sera and other oral fluid samples, initially submitted for antibody testing for MMR surveillance, were stored at -20^{0}C. Oral fluid was collected and extracted from sterile foam swabs (Oracol, Malvern Medical Developments, Worcester, United Kingdom) as described previously (19).

RNA extraction

Total RNA was extracted from clinical specimens using the guanidinium thiocyanate-silica method (16) as described by Jin et al (1996) (81). A detailed description of this method is given in Appendix 3.1 (***pp. 239-230***). Before extraction, foetal tissue was first homogenised in a Griffiths Grinder with Nuclease Free Water (Promega), the tissue sample making up 10% of the final volume. This was followed by two cycles of freeze thawing at -70^{0}C.

RT-PCR

cDNA synthesis

RNA was transcribed to cDNA using Moloney murine leukaemia virus reverse transcriptase and random hexamers as primers as described by Jin et al (81). A detailed description of this method is given in Appendix 3.1 (***pp. 229-230***).

Nested RT-PCR Development and Optimisation

After a series of experiments to optimise the nested RT-PCR performance, as described below, the following procedure was used.

Nested RT-PCR used to amplify a region of the RV E1 gene

Specific primers were chosen for the nested RT-PCR to amplify a 553 nt region of the E1 gene from a 580 nucleotide amplicon produced by a first round reaction. The first round of PCR mixture contained 20ul of cDNA, 10pmol each of primers RE1.1a (5'GTTCCATACAGAGACCAGGA) and RE1.2Ra (5'ACTGGTAGCACCCGGTCACA), 200umol of deoxynucleoside triphosphates (dNTPs), 4.0 U of *Taq*polymerase (Life Technologies, UK), 5ul of 10xPCR buffer (Bioloine UK), 1.5ul of 50mM $MgCl_2$ and made up to 50ul with Nuclease Free Water (Promega). PCR mixture was then overlaid with one drop of light mineral oil. The PCR cycle used was an initial denaturation for 2 min at 95°C followed by 25 cycles of 1 min at 95°C, 1.5 min at 55°C, and 2 min at 72°C. For nested (n) PCR, 10ul of the first round product was added to 25pmol each of primers RE1.3 (5' ACCGTCTGGCAACTCTCCGT) and RE1.4R (5' ACCCGGTCACACGCACATTG), 200umol of deoxynucleoside triphosphates (dNTPs), 4.0 U of *Taq*polymerase (Life Technologies, UK), 5ul of 10xPCR buffer

(Bioloine UK), 1.5ul of 50mM $MgCl_2$ and made up to 50ul with Nuclease Free Water (Promega). After an initial denaturation for 2 min at 95°C, 30 cycles of 1 min at 95°C, 1.5 min at 57.5°C, and 2 min at 72°C were used. 10ul of the nPCR product was analysed on a 2% agarose gel and visualised by ethidium bromide staining.

Primer design

The oligonucleotide primers used in this work were designed for determining the nucleotide sequences of a region of the E1 gene of rubella and were based on published sequence data available in the Genbank. Their design followed some of the recommendations of Saiki (163) and Compton (30), though the limited sequence data available for rubella and high GC content of the rubella genome made this difficult. Each primer sequence was selected within a conserved region of the E1 gene, and the region each primer pair encompassed was designed to be variable. Each primer was 20 nucleotides in length with base compositions that ranged from 50-60% GC. Ideally base compositions should be in the range of 35-55% GC. However, it was not always possible to achieve this due to the particularly high GC content of the rubella genome. All the primers were synthesized by Cruachem Ltd.

Primer concentration

To determine optimal primer concentration the first round primers were used at concentrations of 2.5 pmol/ul, 5 pmol/ul and 10 pmol/ul in conjunction with second round primers at concentrations of 5 pmol/ul, 10 pmol/ul, 15 pmol/ul, 20 pmol/ul and 25 pmol/ul using the positive control (JUD-UK62) grown in vero cells.

Annealing temperature

An optimal annealing temperature for the nested RT-PCR was determined using a temperature gradient consisting of 10 separate temperatures using a Mastercycler Gradient thermocycler (Eppendorf), and the RT-PCR performed using cDNA prepared from the positive control. The temperature gradient used ranged from 55.5°C to 74.8°C for the first round reaction, and from 57.5°C to 76.7°C for the second round reaction.

A dilution series of the positive control from 10^{-1} to 10^{-8}, prepared in Nuclease Free Water (Promega), was used to examine three annealing temperatures in more detail. For the first round reaction annealing temperatures of 55.5°C, 63.4°C and 73.6°C were chosen, and for the second round temperatures of 57.5°C, 65.4°C and 75.5°C.

The primers RE1.1a and RE1.2Ra were used at a concentration of 10 pmol/ul and RE1.3 and RE1.4R used at 25 pmol/ul for these experiments.

Sensitivity of the nested RT-PCR

Once the final conditions of the assay had been optimised, the sensitivity of the nested RT-PCR was assessed in serial dilutions containing estimated copy numbers of cloned first round PCR amplicon, as follows.

The first round PCR product from an oral fluid specimen strongly positive for RV RNA was cloned using the TOPO kit (Invitrogen) according to the manufacturers instructions. Briefly, the first round product was incubated at 72°C for 10 minutes. 2 ul of product, 1ul of cloning vector and 2ul nuclease free water (Promega) were

gently mixed, left to stand at room temperature for 5 minutes and then placed on ice, after which 2ul was added to a vial of competent cells with 2ul of 0.5M *B*mercaptoethanol. This was incubated on ice for 30 minutes and the cells then "heat shocked" for 30 seconds at 42°C without shaking. This was then incubated on ice for 2 minutes after which 250ul of SOC medium (2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) added and the mixture incubated shaking at 37°C for 30 minutes. 100ul was then spread evenly across an agar plate containing 50ug/ml ampicillin and incubated overnight at 37°C. Using a sterile loop, culture from colonies was added to 50ul of PCR mix. After single round PCR using the outer set of primers the presence of the cloned 580 nt RV amplicon was demonstrated by electrophoresis and visualisation of the correct size band on a 2% agarose gel. A detailed method is described in Appendix 3.2 (***pp. 230-232***).

A clone containing the 580 nt RV amplicon was grown overnight at 37°C in 5ml L-broth containing 50ug/ml ampicillin and purified using a combination of reagents from the Quiagen (Quiagen Ltd, Crawley, UK) and Gene-Clean (Bio 101 Inc., USA) kits. In brief, cells were lysed and washed using the buffers from the Quiagen kit and the DNA purified using the "glass milk" (silica matrix) and "New Wash" (a solution of NaCl, Tris, EDTA, ethanol and water designed to maintain a pH of 7.0-8.5) from the Gene-Clean kit (Bio 101 Inc., USA). An estimation of the copies/ul present was made by measuring the absorbance at 260nm (A_{260}) given that 1 A_{260} represents 50ug/ml DNA, that the length of the vector plus the insert is 4.480 kb and that a single copy of 1kb dsDNA weighs 6.5×10^5 daltons ($=1.08 \times 10^{-12}$ ug). Amplification by nested RT-PCR of a dilution series containing cloned copies of the 1st round product

ranging from 0 to 2 x 10^9 in the 20ul volumes added to the first round reaction enabled an estimate of the sensitivity of the nested RT-PCR to be determined by electrophoresis and visualisation of the second round product on a 2% agarose gel after ethidium bromide staining.

TOPO-Cloning exploits the ligation activity of topoisomerase. Since ligation of the vector with a PCR product containing 3' A overhangs is very efficient and occurs spontaneously within 5 minutes at room temperature (169), the manufacturers estimate the efficiency of the kit to be ~95%. The calculations used therefore assume that there is one copy of the inserted RV amplicon per copy of the plasmid.

Specificity of the nested RT-PCR

The specificity of the nested RT-PCR was determined by testing specimens containing other single stranded RNA viruses including hepatitis C virus (HCV), measles virus and polio virus (cDNA was tested) and specimens containing the rash causing DNA viruses parvovirus B19 and human herpes virus 6 (HHV-6).

HCV cDNA was provided by the Hepatitis and Retrovirus Laboratory (HRL), Central Public Health Laboratory (CPHL). Polio cDNA was provided by the Gastroenteritis Unit, ERNVL, CPHL. Measles virus cDNA, parvovirus B19 DNA and HHV-6 DNA were provided by the Immunisation and Diagnosis Unit, ERNVL, CPHL.

"Hot start" technique

Platinum Taq (Pt Taq) DNA polymerase is a recombinant Taq which is complexed with a proprietary antibody which inhibits activity. Pt Taq is provided in an inactive

form and is blocked at ambient temperatures but activity is regained after denaturization at 94°C. This is a "hot start" technique (87) and can help improve PCR specificity by reducing the production of non-specific amplicons.

To evaluate the "hot start" technique RT-PCR was performed using two reaction mixes, one containing 4.0 U of Taq DNA polymerase and the other 4.0 U of Pt Taq DNA polymerase.

Optiprime kit

The Stratagene Opti-Prime PCR Optimisation kit was used in an attempt to improve RT-PCR performance, particularly specificity, using a range of buffers and adjuncts (an incidental component that may be added to any of the buffers used to improve the specificity and/or increase the yield of the desired amplicon). A detailed method is described in Appendix 3.5 (***pp. 235-236***).

Optimal buffer determination - Twelve buffers were available in the Stratagene Opti-Prime PCR Optimisation kit. Each was used including $MgCl_2$ at concentrations of 1.5mM and 3.5mM and KCl at concentrations of 25mM and 75mM. The pH of reaction mixes ranged from 8.3 to 9.2.

Adjuncts - Six adjuncts were included in the first and second round PCR reaction mixes. These included 5ul formamide, 2.5ul DMSO, 7.5ul glycerol, 1ul 750mM $(NH_4)_2SO_4$, 3ul 1.5mg/ml BSA and 0.5ul (=0.5U) Perfect Match DNA Polymerase Enhancer (a commercial product produced by Stratagene which destabilises mismatched primer-template complexes and helps to remove secondary structures which could impede normal extension). The positive control (JUD- UK62) was used

for each adjunct investigated and as a control the positive control (JUD- UK62) was included using the optimised RT-PCR conditions as previously described.

Nucleotide sequence determination

The nested RT-PCR products were excised from agarose gels, purified using a Gene-Clean Kit according to the manufacturers instructions (Bio 101 Inc., USA) and sequenced directly using the internal primers for the RT-PCR. A detailed method for using the Gene-Clean Kit is described in Appendix 3.3 (***pp. 232-233***).

The nucleotide sequences were determined using the *Taq* DyeDeoxy-terminator cycle sequencing kit (Applied Biosystems) with the forward and reverse primers for the nested PCR (RE1.3 and RE1.4R) used at a concentration of 3.2pmol/ul. A detailed method is described in Appendix 3.4 (***p. 234***). The 2',3'-dideoxynucleoside triphosphates are telogens, nucleotides which cause chain termination because they lack a 3' hydroxyl group for extension. The dideoxynucleotides used are labelled with fluorescent dyes. Four dyes are used, one for each base, and each emitts a different wavelength of light. Four sequencing reactions are carried out in parallel, each of which generates nested fragments ending at a defined base and differing in length in steps of a single nucleotide. The side-by-side electrophoresis of these reactions allows the sequence to be read directly from the electrophoresis gel (183).

Computer analysis

Nucleotide sequences were analysed with the SeqEd version 1.0.3 program. A phylogenetic tree was constructed using PAUP 4 Neighbour joining software with 1000 bootsraps. Bootstraping is a technique for estimating the variance and the bias of

an estimator by repeatedly drawing random samples with replacement from the observations at hand (91).

RV antibody detection

Oral fluid or serum samples were tested for RV specific IgM antibody by antibody capture radioimmunoassay as previously described (144). Results were expressed as a test:negative ratio (T:N) and specimens considered reactive for RV IgM if they gave a T:N greater than 3.0. All serological testing of sera and oral fluid samples used in this part of the study was carried out by staff of the Immunisaton and Diagnosis Unit, ERNVL, CPHL and the results kindly made available.

Investigating oral fluid as a specimen for detecting virus genome

This section of the study was designed to investigate the extent to which oral fluid specimens may be used for detecting virus genome. This involved comparing the results of oral fluid samples subjected to measles, mumps and rubella RT-PCR collected from patients with clinical or serological evidence of these infections at different intervals after the onset of symptoms.

A total of 1047 oral fluid samples were used in this part of the study. All were collected using the Oracol device (Malvern Medical Developments) and oral fluid extracted as previously described (19). All oral fluid samples were collected as part of the MMR surveillance programme in the UK (62, 63, 111) and had previously been screened for IgM to either measles, mumps or rubella (144) with the exception of 55 which were obtained specifically for measles RT-PCR and 26 specifically for rubella RT-PCR. These samples were collected from patients with clinical evidence of these infections, from a rural district of Ethiopia and a Thessaloniki in northern Greece respectively (kindly provided by Professor V. Kyriazopoulou-Dalaina). They were cryopreserved and only made available for virus specific IgM testing ***after*** they had been screened by RT-PCR. All other samples were stored at -20^{0}C and had been subjected to at least one freeze/thaw cycle prior to being used for RT-PCR testing. The period, in days, between onset of symptoms and timing of sample collection was recorded and the samples classified into categories of 0-7 days, 8-14 days or > 14 days after onset of symptoms. A "not known" category was used to classify samples where the date of onset or date of sample collection had not been recorded.

Virus genome was extracted using the guanidinium thiocyanate-silica method as described by Boom et al (16) and reverse transcribed to produce cDNA as previously described (81) using the method described in Appendix 3.1. The 553 nucleotide region of the E1 gene of the rubella virus genome was then amplified by nested RT-PCR as previously described, and regions of the measles (N and M) and mumps (SH) genes amplified by specific nested RT-PCR as described by Jin et al (79, 81) Detailed methods are described in Appendix 3.6 and 3.7 (***pp. 237-238***). All three RT-PCR methods produce second round amplicons of a size suitable for both diagnosis and molecular epidemiology. The second round amplicons were visualised by staining with ethidium bromide after electrophoresis on a 2% agarose gel, and results classified as positive or negative depending upon the presence or absence of the appropriate sized bands. The results of mealses and mumps nested RT-PCR assays were kindly provided by Dr Li Jin (Immunisation and Diagnosis Unit, Enteric, Respiratory and Neurological Virus Laboratory, CPHL), the laboratory work having been carried out by Mr Stuart Beard and Mr Rashpal Hunjan (also of the Immunisation and Diagnosis Unit). The analysis of RT-PCR results in relation to the timing of sample collection was subsequently carried out in this study.

Statistical Methods

Logistic regression was used to compare rates of detection of measles, mumps and rubella virus genome with time after onset of symptoms (5).

RESULTS

The development of a PCR assay suitable for the molecular epidemiology of RV and assisting with diagnosis

RT-PCR optimisation

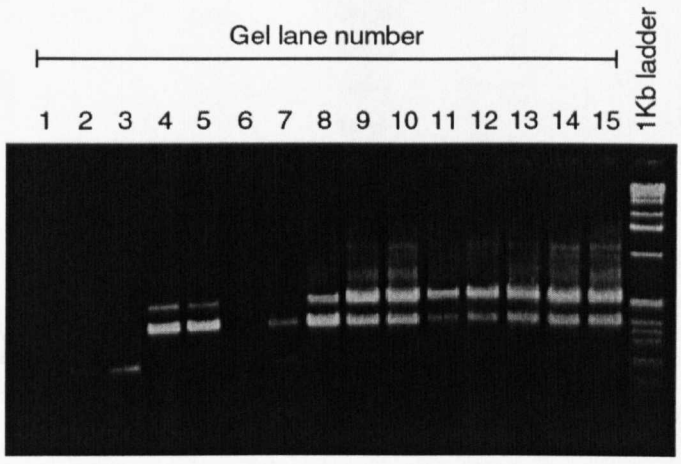
The results of varying the concentrations of both the first and second round primers are shown in Table 28 and Fig. 27.

Table 28. nested RT-PCR result with varying primer concentration

1st round primer conc. (pmol/ul)	2nd round primer conc. (pmol/ul)				
	5	10	15	20	25
2.5	NEG [1]*	NEG [2]	NEG [3]	POS [4]	POS [5]
5	NEG [6]	NEG [7]	POS [8]	POS [9]	POS [10]
10	POS [11]	POS [12]	POS [13]	POS [14]	POS [15]

*[_] refers to gel lane number in Fig. 27

Figure 27: 2% Agarose gel of nested RT-PCR products produced with varying primer concentration (see Table 28)



*The larger of the two products seen is the 553 nt RV specific amplicon

The results showed that the lower the first round primer concentration the higher the second round primer concentration needed to successfully amplify the target and produce a positive result. Therefore a first round and second round primer concentration of 10 pmol/ul and 25 pmol/ul respectively was chosen. At these concentrations primers were present in excess and primer concentration was not a limiting factor in RT-PCR sensitivity. These concentrations have also been successfully used for measles and mumps RT-PCR (79, 81).

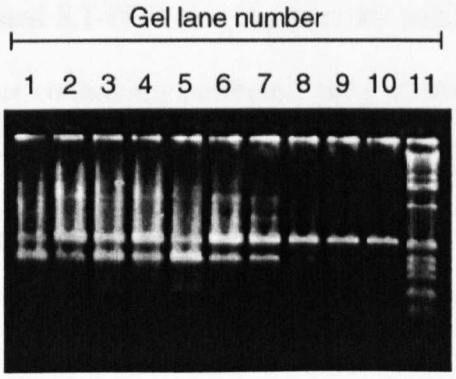
Annealing Temperatures

Tables 29, 30, and Fig. 28 show the effect of altering the annealing temperatures for both the first and second round nested RT-PCR reactions.

Table 29. Gel lane and corresponding annealing temperature (see Fig. 28)

Gel Lane	ANNEALING TEMPERATURE (^{0}C)	
	1st round	2nd round
1	55.5	57.5
2	56.5	58.5
3	58.3	60.3
4	60.7	62.7
5	63.4	65.4
6	66.3	68.3
7	69.1	71.1
8	71.6	73.6
9	73.6	75.5
10	74.8	76.7
11 (1 kb ladder)	-	-

Figure 28: RT-PCR result and corresponding annealing temperature (see Table 29)



* The larger of the two products seen is the 553 nt RV specific amplicon

Table 30. Nested RT-PCR result at three annealing temperatures for a dilution series of the positive control.

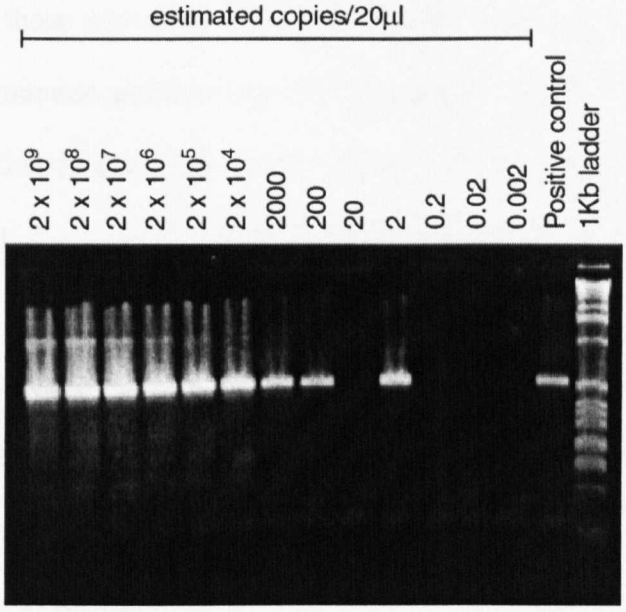
Dilution of POS control	Annealing temperatures (^{0}C) : 1st round [2nd round]		
	55.5 [57.5]	63.4 [65.4]	73.6 [75.5]
10^{-1}	POS	POS	POS
10^{-2}	POS	POS	POS
10^{-3}	POS	POS	NEG
10^{-4}	POS	POS	NEG
10^{-5}	POS	NEG	NEG
10^{-6}	NEG	NEG	NEG
10^{-7}	POS	NEG	NEG
10^{-8}	NEG	NEG	NEG

These results showed that RT-PCR sensitivity was highest using annealing temperatures of 55.5^{0}C and 57.5^{0}C for the first and second round reactions respectively.

Sensitivity of the nested RT-PCR

The sensitivity of the nested RT-PCR was assessed by testing the cloned first round PCR amplicon at dilutions containing estimated copy numbers from $2x10^9$ to $2x10^{-3}$ copies. The nested RT-PCR was able to detect rubella genome in dilutions containing between 1 and 10 copies of target (Table 31, Fig. 29).

Figure 29: Sensitivity of the nested RT-PCR



No rubella genome was detected by the RT-PCR in the dilution estimated to contain 20 copies of rubella genome in the 20ul added to the first round reaction, though genome was detected in that estimated to contain 2 copies. This may be a result of poor preparation of the original dilution series or innaccurate pipetting when transfering target at this particular concentration to the first round reaction which could result in no copies of the genome being transfered to the first round reaction and therefore give a negative result. Alternatively, this may be due to the probability that no copies are transfered when small volumes that only contain a very low concentration of the target at the end point of the dilution series are transfered by pipetting.

Specificity of the nested RT-PCR

The specificity of the rubella nested RT-PCR was established by testing specimens containing genome of other rash causing virus infections (parvovirus B19, measles and HHV-6) and those containing cDNA from polio virus and HCV, both of which possess a single stranded positive sense RNA genome with HCV having a similar genomic organisation to that of RV. No cDNA or DNA molecules were amplified (results not shown).

Two of the 219 oral fluid samples tested by RT- PCR produced an amplicon of weak intensity that could not be visually distinguished in size from the 553 nucleotide rubella specific amplicon produced by the nested reaction, though no specific rubella gene sequence was identified (Fig 30). The presence of amplicons other than the 553 nucleotide rubella specific amplicon produced by the nested reaction, in particular one approximately 350 nucleotides in size (Fig 30), were also seen with a large proportion of the samples. These were later confirmed to be parts of human genome by direct sequencing.

Hot start - No improvement in the nested RT-PCR specificity was observed when comparing Pt Taq DNA polymerase and Taq DNA polymerase (results not shown).

Stratagene Opti-Prime PCR Optimisation kit - No improvement in nested RT-PCR specificity was observed by using any of the buffers or adjucts included in the Stratagene Opti-Prime PCR Optimisation kit (results not shown).

RT-PCR detection of RV genome in clinical specimens

All 49 serum samples and 214/219 oral fluid samples were positive for RV specific IgM, indicating recent infection. Of the five oral fluid samples which were RV IgM negative, four were positive by nested RT-PCR and all five were collected $\leq$ 2 days after the onset of symptoms.

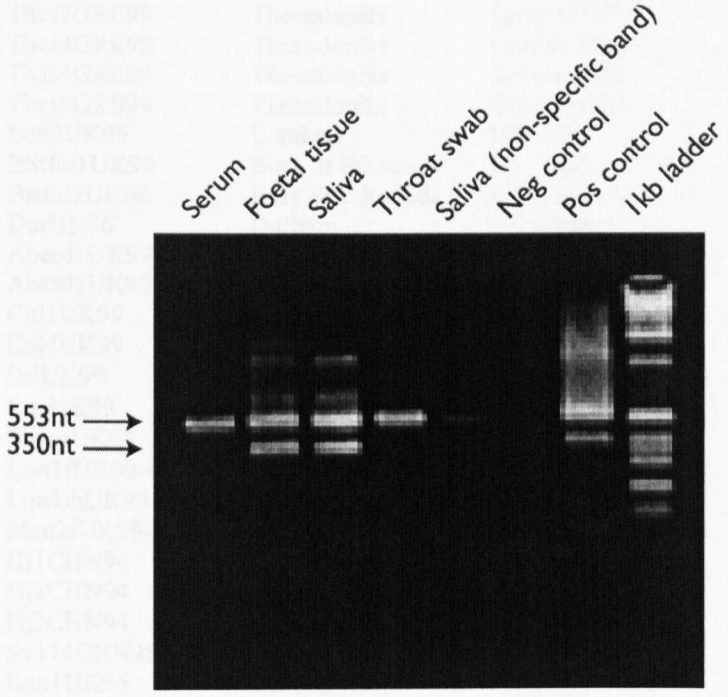
The proportion all samples PCR positive for RV RNA are shown in Table 31.

Table 31. Samples tested by PCR for RV RNA

Specimen	Collection purpose	Total	RT-PCR POS (%)	Period: onset of symptoms to sample collection (where known)		
				n	mean (days)	range (days)
Serum	Antibody detection	49	6 (12.2)	-	-	-
Saliva	Antibody detection	200	14 (7)	147	20.1	1-56
Saliva	RT-PCR	19	13 (68.4)	19	5.1	1-12
Throat swab	RT-PCR	41	26 (63.4)	32	4.1	0-13
Foetal Tissue	RT-PCR	2	2 (100)	-	-	-
TOTAL		311	61 (19.6)			

The results obtained from the four different types of specimen used in this study which were oral fluid, serum, throat swab, and foetal tissue are illustrated in Fig. 30. The non specific bands of 350 and 553 nucleotides in size were described above.

Figure 30: Agarose gel analysis of RV RT-PCR products from a range of clinical specimens



Of 49 sera and 200 oral fluid samples originally collected for antibody detection, 12.2% and 7% respectively were positive by RT-PCR, as were 13/19 (68.4%) oral fluid samples collected specifically for RT-PCR (Table 31). Overall 12.3% oral fluid samples were positive by RT-PCR. A total of 26 (63.4%) throat swabs and 2 (100%) foetal tissue samples were positive by RT-PCR. The geographical location of representative samples is listed in Table 32.

Table 32. Origin of samples directly sequenced

Strain name[1]	City/province	Country (OB/Spor)[2]	Year collected	Specimen type
Sy2CHN99	Shenyang city	China (Spor)	1999	Throat swab
Sy7CHN99	Shenyang city	China (Spor)	1999	Throat swab
Thes1GRE99	Thessaloniki	Greece (OB)	1999	Oral fluid
Thes2GRE99	Thessaloniki	Greece (OB)	1999	Oral fluid
Thes3GRE99	Thessaloniki	Greece (OB)	1999	Throat swab
Thes4GRE99	Thessaloniki	Greece (OB)	1999	Throat swab
Thes5GRE99	Thessaloniki	Greece (OB)	1999	Throat swab
Lon2UK96	London	UK (OB)	1996	Oral fluid
BStEd1UK96	Bury St Edmunds	UK (OB)	1996	Oral fluid
BstEd2UK96	Bury St Edmunds	UK (OB)	1996	Oral fluid
DurUK96	Durham	UK (OB)	1996	Oral fluid
Aberd1UK99	Aberdeen	UK (Spor)	1999	Oral fluid
Aberd2UK99	Aberdeen	UK (Spor)	1999	Oral fluid
Col1UK99	Colchester	UK (Spor)	1999	Oral fluid
Col4UK99	Colchester	UK (Spor)	1999	Oral fluid
SalUK99	Salford	UK (Spor)	1999	Oral fluid
LonUK99	London	UK (Spor)	1999	Oral fluid
ShropUK99	Shropshire	UK (Spor)	1999	Oral fluid
Lon1fUK99-CRS[3]	London	UK (Spor)	1999	Foetal tissue (Foetus)
Lon1mUK99-CRS[3]	London	UK (Spor)	1999	Serum (Mother)
Man2fUK99-CRS[3]	Manchester	UK (Spor)	1999	Foetal tissue (Foetus)
Hj1CHN94	Heilong jiang prov.	China (Spor)	1994	Serum
Hj2CHN94	Heilong jiang prov.	China (Spor)	1994	Serum
Hj3CHN94	Heilong jiang prov.	China (Spor)	1994	Serum
Sy114CHN98	Shenyang city	China (Spor)	1998	Serum
Lon1UK96	London	UK (OB)	1996	Oral fluid
NyorkUK96	North Yorkshire	UK (OB)	1996	Oral fluid
Rio1BRAZ99	Rio de Janiero	Brazil (Spor)	1999	Serum

[1] _____ (City/province)__(sample no.)____(Country)__(Year collected); [2] OB = outbreak, Spor = sporadic case; [3]congenital rubella

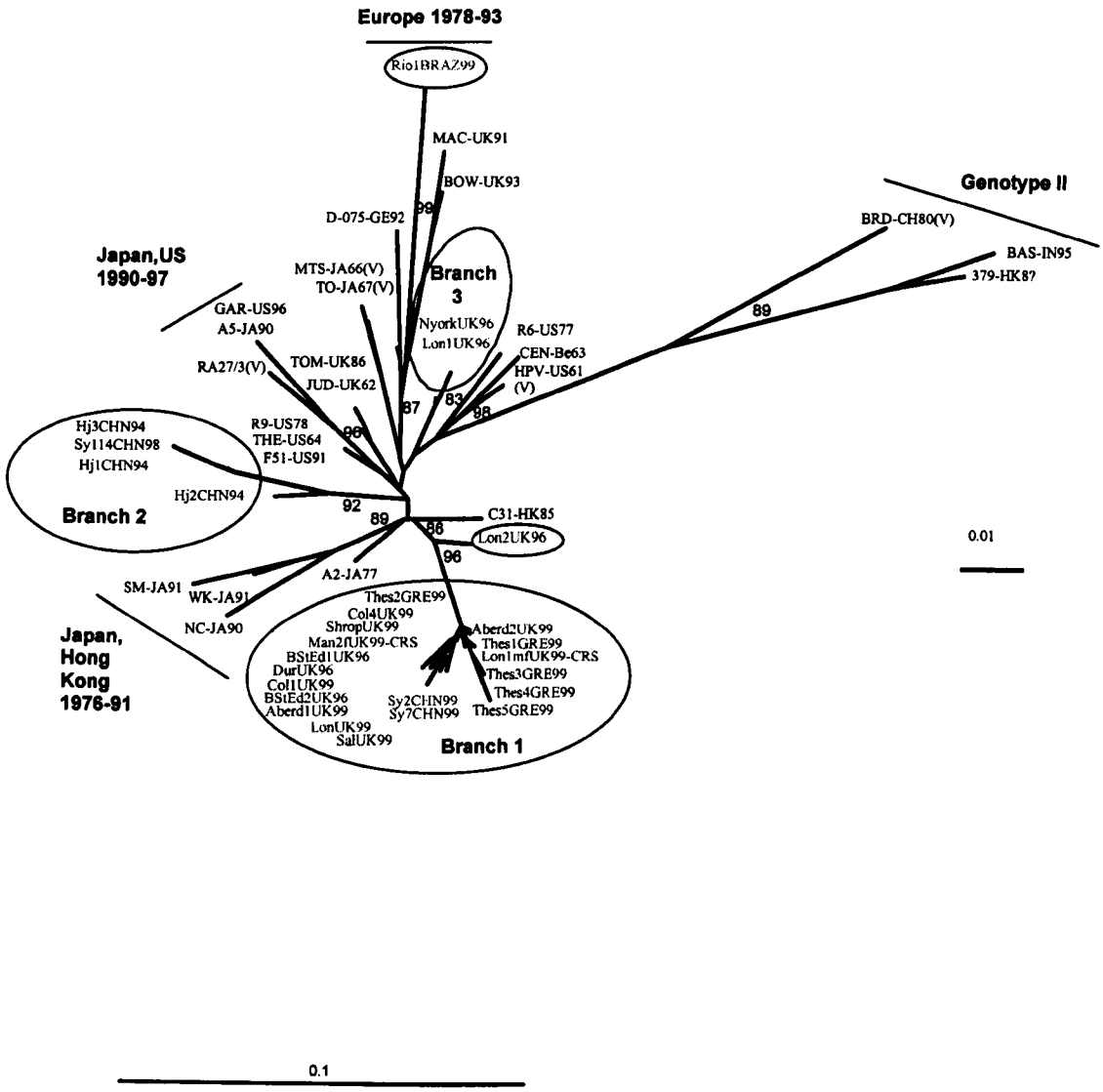
The interval between onset of symptoms and sample collection was recorded for 147 of the 200 oral fluid specimens originally submitted for antibody detection, for 32 of 41 throat swabs and for all 19 oral fluid specimens specifically collected for RT-PCR (Table 32). For the 147 oral fluid samples originally submitted for antibody detection the mean period between onset of symptoms and sample collection was 20.1 days, ranging from 1 to 56 days, whilst for the 19 oral fluid specimens collected for RT-PCR the mean period between onset of symptoms and sample collection was 5.1 days,

ranging from 1 to 12 days. For the 32 throat swabs the mean period between onset of symptoms and sample collection was 4.1 days, ranging from 0 to 13 days.

Genetic characterisation of RV strains

Of the 61 samples that were positive by RT-PCR, 28 were directly sequenced using the internal primers for the nested PCR reaction. The geographical location and other relevant information is listed in Table 32. Figure 31 shows a phylogenetic tree with 28 sequences from samples used in this study and 24 sequences from previously reported data (57).

Figure 31: Genetic relationship between rubella virus strains used in this study and previously reported strains



***Circled strains were used in this study. The numbers indicate bootstrap values**

__x__ = genetic distance

The samples used in this study clustered to form three branches on the phylogenetic tree. Those clustered on Branch 1 consisted of 14 samples from the UK collected in 1996/99, 5 samples from Greece collected during an outbreak in 1998/99 and two sporadic cases from China obtained in 1999. Branch 2 consisted of 4 samples from sporadic cases that occured in China in 1994/98. Two strains obtained in the UK in 1996 (Lon1UK96 and NyorkUK96) were grouped on Branch 3. The strain obtained from a sporadic case in Rio de Janiero, Brazil, in 1999 (Rio1BRAZ99), is similar to those from the branch previously described as Europe 1978-93 by Frey et al (57).

The greatest divergence in nucleotide sequence, 12.0%, was seen when comparing strain Rio1BRAZ99 (Europe 1978-93) from this study to the BASInd95 strain (Genotype II) isolated in India in 1995 (17, 57). The single sequence analysed from China collected in 1998 (Sy114CH98) was more closely related to the three isolates collected in China in 1994 (Branch 2) than to those isolated in 1999 (Branch 1), which had very similar nucleotide sequences to those from Europe obtained in 1996 and 1999 grouped on Branch 1. Sequences analysed from European strains obtained in 1999 forming part of Branch 1 showed little divergence in nucleotide sequence, differences ranging from 0.2% - 1.6%. The majority of samples from the UK in 1996 used in this study also showed little difference in nucleotide sequence compared to those from the UK in 1999 (0.2 - 2.6%). However, the two samples collected in 1996 that formed Branch 3 - Lon1UK96 and NyorkUK96 - were more closely related to the standard laboratory strain (JUD-UK62) isolated in Liverpool in 1962 with a 0.4% difference in nucleotide sequence than to others from 1996, from which they showed a 2.0 - 3.8% difference in nucleotide sequence.

The E1 gene encodes 412 amino acids and the RT-PCR assay described amplifies a region encoding 171 of these, from amino acid 171 to 342 (25). Of the samples analysed by direct sequencing in this study, the maximum number of amino acid changes observed compared to the concensus sequence was three (1.8%), with no more than a single amino acid change being observed between amino acids 214-285.

Oral fluid as a specimen for detecting virus genome

A total of 1047 oral fluid samples tested by RT-PCR for measles, mumps and rubella were analysed in this study and the results for each virus relative to time of sample collection are shown in Table 33.

Measles

A total of 448 oral fluid samples were screened for measles virus genome and were categorised into those previously tested for specific IgM for surveillance and those which were collected specifically for RT-PCR from persons with symptoms of measles. These were mostly obtained within 7 days of the onset of symptoms (Table 33). Of 393 oral fluid samples previously tested for antibody, all contained measles specific IgM and the period between collection of sample and onset of symptoms was known for 293 (74.6%). Measles virus genome was detected in 53.8%, 40.3% and 20.7% of those collected in the first week, second week and after the second week of onset of symptoms respectively. Of 55 oral fluid samples collected specifically for RT-PCR from patients clinically diagnosed as having acute measles infection, 54 were collected during the first week after onset of symptoms with measles virus genome being detected in 52 (96.3%). Measles virus genome was also detected in the single oral fluid sample collected in the second week after the onset of symptoms.

Mumps

Of 354 oral fluid samples screened for mumps virus genome, the period between collection of sample and onset of symptoms was known for 305 (86.2%). Mumps virus genome was detected in 68.7%, 20% and 1.6% of those collected in the first

week, second week and later than two weeks after the onset of symptoms respectively. All samples had previously been tested for antibody and all contained mumps specific IgM.

Rubella

A total of 245 oral fluid samples were tested for rubella virus genome. As for measles, these samples were categorised into those previously tested for specific antibody for surveillance and those which were collected specifically for RT-PCR from persons with symptoms of rubella. Of the 219 oral fluid samples previously tested for antibody, rubella specific IgM was detected in 214 (98%) and the period between collection of sample and onset of symptoms was known for 156 (71.2%). Rubella virus genome was detected in 41.7%, 4.2% and 2.8% of those collected in the first week, second week and after the second week of onset of symptoms respectively. Of a total of 26 oral fluid samples specifically collected for RT-PCR from patients clinically diagnosed as having acute rubella, virus genome was detected in 75% of those collected within a week of the onset of symptoms and in 30% of those collected in the second week after the onset of symptoms.

Table 33. Analysis of measles, mumps and rubella RT-PCR results by time from onset of clinical symptoms on 1047 oral fluid samples from serologically and clinically confirmed cases

Virus	Collection purpose	RT-PCR Result	Days after onset (% +ve)				TOTAL
			0-7	8-14	>14	Not Known	
Measles	Antibody detection	Positive	28 (53.8)	27 (40.3)	36 (20.7)	19 (19)	110 (28)
		Negative	24	40	138	81	283
		TOTAL	52	67	174	100	393
	RT-PCR	Positive	52 (96.3)	1 (100)	-	-	53 (96.4)
		Negative	2	0	-	-	2
		TOTAL	54	1	-	-	55
Mumps	Antibody detection	Positive	46 (68.7)	11 (20.0)	3 (1.6)	4 (8.2)	64 (18.1)
		Negative	21	44	180	45	290
		TOTAL	67	55	183	49	354
Rubella	Antibody detection	Positive	10 (41.7)	1 (4.2)	3 (2.8)	2 (3.2)	16 (7.3)
		Negative	14	23	105	61	203
		TOTAL	24	24	108	63	219
	RT-PCR	Positive	12 (75.0)	3 (30.0)	-	-	15 (57.7)
		Negative	4	7	-	-	11
		TOTAL	16	10	-	-	26

DISCUSSION

The development of a RT-PCR assay suitable for molecular epidemiology and assisting with RV diagnosis

The aim of this aspect of the study was to develop a RT-PCR assay that is useful for diagnostic purposes and which also allows subsequent sequencing of the amplicons for epidemiological comparisons of different RV strains. PCR primers were selected from the E1 gene because humoral responses are directed mainly against the E1 glycoprotein. E1 is more exposed than E2 and studies with monoclonal antibodies showing that neutralizing epitopes reside mainly on E1 (56, 86, 114, 195). A region of the E1 gene was selected which is sufficiently conserved so that all known genotypes would be detected but is also suitable for distinguishing between different genotypes. The primers encompassed a region which codes for amino acids 245-285 that have been shown to be antigenically important (56). The nested product was designed to be of sufficient length (553nt) to provide useful sequence data.

PCR assays have been described previously for detecting RV RNA (18, 155, 175). However, their application for genetic characterisation has been limited as most had been specifically designed for diagnostic purposes. To date there have been three other studies analyzing RV sequences, both targeting the entire E1 gene. The first was described by Bosma et al and used 22 rubella virus isolates from Europe, USA and Asia obtained between 1963-1995 (17).That described by Katow et al (86) used samples from three east Asian countries whilst Frey et al (57) conducted a more extensive study of the molecular epidemiology of RV using samples from the continents of North America, Europe and Asia collected between 1961 and 1997. Their results suggested that two genotypes existed with nucleotide sequences that

differed from each other by 8% - 10%. However, no major antigenic variation was observed and only a small proportion of those RV strains sequenced were categorised as genotype II.

The primers used in this study successfully amplified the cDNA target sequence of the control RV RNA (JUD-UK62 strain) and of some clinical specimens. Experiments showed the nested RT-PCR assay was most sensitive when using annealing temperatures of 55.5^{0}C and 57.5^{0}C respectively for the first and secound round reactions. The assay detected between 1 and 10 copies of target (Fig. 29, Table 31). This level of sensitivity is similar to that described by RT-PCR assays for other virus infections (18, 81).

The specificity of the assay was assessed using samples containing genome of other rash causing virus infections (parvovirus B19, measles, HHV-6) and those containing cDNA from polio and HCV. No amplicon was produced from these samples suggesting that the RV RT-PCR assay is not subject to cross reaction. However, two of the 311 clinical specimens used in this study produced an amplicon which appeared visually to be similar in size to that of the positive control by gel detection (Fig. 30). An explanation for this may be the unusually high proportion of guanosine and cytidine found in the rubella genome (69.5%), a phenomenon common to the human genome and a feature which makes the design of appropriate rubella primer sites difficult. In clinical samples, such as oral fluid specimens and tissue samples in particular, it is likely that human genome will also be present. The primers may therefore bind to and amplify certain regions of the human genome and in some cases produce an amplicon of similar size to that of the desired nested product. This idea

was supported by an internet blast search of the rubella primer sequences used in this study in comparison with all available genome sequences. The primers showed a homology of >90% with certain regions of the human genome. Further evidence was provided by direct sequencing of the amplicon approximately 350 nucleotides in length, often seen migrating further than the 553 nucleotide rubella specific amplicon produced by the nested reaction (Fig. 30). Sequence analysis, in combination with a blast search on the internet, showed it to match a region of the human genome. Furthermore, none of the extensive range of buffers or adjuncts provided with the Stratagene Opti-Prime PCR Optimisation kit improved the specificity of the RT-PCR and no improvement was seen by employing a hot start technique. However, amplicons that appear to be true positives can be confirmed by direct sequencing but this approach is not cost effective. An alternative method could involve hybridisation of amplicons with a RV specific probe. However, for this method to be feasible more extensive sequence data is required for RV to enable the design of an appropriate probe that is useable with all strains of RV.

It might be possible to improve the specificity of the RT-PCR by increasing the annealing temperatures for both the first and second round reaction because primer sequences bind to their complementary target sequence with a specificity that increases with temperature. The major problem with this approach is that sensitivity decreases with increasing annealing temperature so such a solution should be viewed with caution.

All serum samples and the majority of oral fluid specimens (214/219 [98%]) used in this study showed serological evidence of recent RV infection by the presence of

specific IgM. Of five oral fluid samples which did not contain RV specific IgM, RV genome was detected in four and all five were collected $\leq$ 2 days after the onset of symptoms. This suggests that when these samples were collected sufficient time had not elapsed for a suitable IgM reponse to have been mounted (69). The fifth oral fluid sample which did not contain RV specific IgM and in which RV genome was not detected may represent a case that was wrongly diagnosed as rubella, or the virus genome may have degenerated due to either cycles of freeze-thawing or being kept for a period at an inapproprate temperature (as described below).

Although not comprehensively investigated, some useful information about suitable samples for RV RT-PCR was identified. In discussing this it is useful to divide the samples into two categories: those that were collected specifically for RV RT-PCR and those used first for RV IgM tests and only then made available for RV RT-PCR. Those collected specifically for the purpose of RV RT-PCR were cryopreserved at -70^{0}C until required for testing whilst samples obtained for RV IgM tests were stored at -20^{0}C and had been subjected to at least one cycle of freeze-thawing before being tested by RV RT-PCR.

A proportion of oral fluid samples used (9%) and all throat swabs were collected and cryopreserved specifically for RT-PCR at -70^{0}C until required for testing. These were found to be the most reliable specimens for detecting RV RNA by PCR. This may reflect the fact that these samples were stored under conditions most likely to preseve virus genome and had not been subjected to cycles of freeze-thawing for other laboratory tests prior to RT-PCR. In contrast to these samples, all sera and the majority of oral fluid samples (91%) were collected for RV IgM testing and had

therefore been subjected to cycles of freeze-thawing before becoming available for RT-PCR. They were also stored at -20^{0}C, a temperature which may be less efficient than cryopreservation at -70^{0}C for preserving virus genome. RNA is a very labile molecule and prone to the degradative effects of any nucleases - such as ribonuclease (RNase) - that may be present in clinical samples. Coupled to freeze-thawing, this is likely to reduce the likelihood of detecting RV genome in a sample. This is supported by the observation that of those oral fluid samples collected specifically for RT-PCR from suspected cases of rubella, 13/19 (68.4%) were positive for RV genome by PCR in contrast to 14/200 (7%) of those previously used for RV IgM testing (Table 24). Therefore storage temperature and cycles of freeze-thawing are factors which may be effecting the likelihood of detecting RV genome in a sample by nested RT-PCR and should be investigated further.

A third consideration is the period between onset of disease and sample collection. The detection rate was shown to decline with increasing time between onset and sampling (Table 32). For instance, oral fluid samples originally obtained for RV IgM testing were collected a mean of 20.1 days after the onset of symptoms (range 1-56 days) and only 7% were RT-PCR positive. In contrast, oral fluids and throat swabs obtained specifically for RT-PCR were collected a mean of 5.1 (range 1-12 days) and 4.1 days (range 0-13 days) respectively after the onset of symptoms and 68.4% and 63.4% were RT-PCR positive. Therefore, it is possible that the length of period between onset of disease and sample collection may be proportional to the likelihood of detection of RV genome. Reasons for this observation and the suitability and extent to which oral fluid specimens may be used for detecting RV genome and other virus

genomes - particularly in relation to the timing of their collection - is discussed in detail later (pp. 223-228).

These data therefore suggest that if appropriately collected and stored, oral fluid is a suitable non-invasive specimen to use for RV RT-PCR. Further types of specimen should also be investigated for the detection of RV RNA and include urines and nasopharyngeal aspirates (NPA).

Foetal tissue from two pregnancies terminated due to suspected CRS, were positive by RV RT-PCR and confirmed by direct sequencing. This suggests that the assay described here may be useful for the diagnosis of CRS. The assay, however, needs to be more fully evaluated using amniotic fluid or chorionic villus samples from suspected cases of CRS if it is to be used for pre-natal diagnosis.

Genetic analysis of RV strains

The most extensive study of the phylogeny of RV to date was carried out by Frey et al in 1998 (57) using specimens collected from North America, Europe and Asia between 1961-1997. Two genotypes were described (Genotype I and II). A proportion of those categorised as Genotype I were classified into three branches that were geographically and chronologically distinct (57). The divergence seen between Genotype I and II ranged from 8.1% to 12%. The divergence between the three branches identified within Genotype I (57) ranged from 2.8% to 6.5%.

In the present study, 61 specimens collected between 1994 and 1999 were positive for RV RNA by RT-PCR. For the purpose of epidemiological investigation 28

representative specimens from each country included in the study were analysed by nucleotide sequencing. All 28 were classified as Genotype I and clustered on a phylogenetic tree to form at least three new branches when compared with previously reported strains (Fig. 31). Strains grouped on branch 1 showed a divergence ranging from 2.0% to 7.0% compared to others classified as Genotype I, and from 8.6% to 10.8% to strains classified as Genotype II (57). Strains grouped on branch 2 showed a divergence ranging from 3.0% to 7.3% in comparison to others classified as Genotype I, and from 9.3% to 11.8% to those classified as Genotype II (57). Two strains obtained in the UK in 1996 (Lon1UK96 and NyorkUK96), grouped on Branch 3, and were most closely related (0.4% divergent) to the standard laboratory strain isolated in Liverpool, UK, in 1962 (JUD-UK62) (57). This suggested that RV strains in Branch 3 may represent an indigenous strain that has been circulating in the UK since the early 1960s. The Brazilian strain investigated in this study, Rio1BRAZ99, was most closely related (4.9% - 5.8% divergent) to previously reported strains obtained from Europe in 1978-93 that formed a separate branch of Genotype I (57) (Fig. 32). This strain could be the result of an importation into Brazil. It was the only strain from this study to be clearly classified in a previously reported branch. A further strain collected in the UK in 1996 (Lon2UK96) could not be classified on any existing branches and the degree of divergence (1.8% - 5.9%) from all other strains of Genotype I suggested that it may form an additional branch if similar strains are identified. The results of this study therefore suggest that it may be possible to group and classify RV strains further than has previously been described.

The last resurgence of RV infection in the UK occurred in 1996 (111). The majority of confirmed cases were young adult males who had never been offered rubella

vaccine and outbreaks occured nationwide, especially in residential establishments such as university campuses (111). That RV sequences analysed in samples collected from the UK during this period were grouped in two branches of the phylogenetic tree (Branch 1 and Branch 3) and that a further strain (Lon2UK96) may form a new branch suggests that more than one strain of RV was circulating in this outbreak. There was little difference in nucleotide sequence (0.4 - 1.8%) for RV sequences analysed from Greece and the UK collected in 1999. Cases of rubella reported in the UK during 1999 are likely to be the result of importations from the outbreak which occured in Greece during 1998/99 (136, 154) since the majority of confirmed cases were university students who were Greek or had contact or connections with Greek students (181, 188). The two samples from sporadic cases in China (Sy2CH99, Sy7CH99) obtained in 1999 also had a very similar nucleotide sequence (1.0% - 2.0%) to those circulating in the UK and Greece at this time and may therefore be the result of an importation from Europe.

Very little difference in the amino acid composition derived from nucleotide sequences - particularly between amino acids 214 to 285 - was found for the samples analysed. This suggests that RV is not prone to major antigenic variation within the E1 gene and supports the findings of other studies (57, 86) that there is little or no antigenic variation within the E1 gene. Explanations for the small amount of sequence variation within the region of the E1 gene studied may be that it is assisting the virus to optimise its replication. Alternatively, the small amount of variation seen may be due to errors that occur during RNA replication (56).

This study highlights the need for further work using specimens from areas of the world that are as yet unsampled such as Africa, Eastern Europe and Australia. this needs to be supplemented with further isolates from those regions that are currently under represented including South America, India, and China. This will help determine the geographic extent of RV strain variation and whether other genotypes exist. Sequencing the entire RV genome from a variety of strains will also confirm which genomic region is most suitable to target for molecular epidemiological studies.

The role of oral fluid as a clinical specimen for detecting virus genome

The suitability of oral fluid for the detection of virus genome was investigated to a limited extent for RV in the work described in the previous section of this chapter on the development of the RV specific RT-PCR. In this section, the role and usefulness of oral fluid for the detection of virus genome is described more extensively for measles and mumps as well as for rubella. However, as mentioned in the introduction to this part of the study, this work is not exclusive to measles, mumps and rubella and could be extended to include other virus infections since a range of viruses have been detected in oral fluid (1, 22, 38, 78, 83, 98, 99, 162, 176).

In interpreting the relationship between measles, mumps and rubella RT-PCR results and time from onset of clinical symptoms it was assumed that all samples were obtained from cases correctly diagnosed as measles, mumps or rubella. That 966/1047 (92%) of the oral samples were shown to contain either measles, mumps or rubella specific IgM and had therefore been collected from persons who had recently been infected by these diseases supports this assumption. The remaining 81 (8%) samples were collected from persons who were clinically diagnosed as having acute measles or rubella, and whilst serological data was not available on all these samples, measles or rubella genome was detected in 68 (84%) samples suggesting the diagnoses were reliable.

The analysis performed in this study has shown that meales, mumps and rubella virus genome can be reliably detected in oral fluid samples by RT-PCR from patients with evidence of recent infection. The most important factor affecting the success of this approach was the timing of sample collection since viral RNA levels decline after the

onset of disease. The results (Table 34) indicated that RT-PCR can detect viral genome in acute oral fluid specimens collected for early diagnosis whereas viral IgM may not be detectable until several days after onset of symptoms, the optimal timing of collection for this purpose being two to three weeks after onset of symptoms (69).

There are several reasons why measles, mumps and rubella virus may be detected in oral fluid. The close proximity of the oral cavity to the respiratory tract may contribute, the respiratory tract being a portal of entry and initial site of replication for all three viruses before spreading to lymph nodes, subsequent viraemia and spread to other tissues. Once viraemia has been established it is also possible that there may be transudation of virus from the blood to the crevicular fluid via the capillary beds in the gingival crevice. Characteristic symptoms of measles and mumps infection are associated with the oral cavity and this may also contribute to the presence of the virus at this site. Measles virus has been cultured from the mouth and Koplik's spots, visible on the buccal mucosa, often coincide with the first symptoms of disease (67). Swelling of the salivary glands (parotitis) is a common feature of mumps infection and suggests that mumps virus may invade and be shed from these glands (194).

Factors which lead to the decline in viral RNA levels after the onset of disease include virus clearance by the immune system and RNA degeneration. The length of the period between onset of disease and sample collection is proportional to the extent of the immune response mounted by the host to clear the virus and thus inversely proportional to the amount of virus present. Since this is a biological phenomenon important for the limitation of disease it is not therefore surprising that the likelihood

of detecting each virus genome was shown to decrease as the period between onset of symptoms and sample collection increased.

It is well established that RNA is a labile molecule and is decayed by such degradative nucleases as ribonuclease (RNase) which are likely to be present in oral fluid and may be human, bacterial or viral in origin (125). Therefore it is likely that the period between onset of disease and sample collection will be inversely proportional to the amount of virus genome present. This argument can be extended to include the handling of samples prior to RT-PCR. Subjecting an oral fluid sample to cycles of freeze thawing for other laboratory purposes such as antibody detection proir to RT-PCR, is likely to increase virus genome exposure to any RNase that may be present in the sample The likelihood of detecting virus genome is decreased because enzyme activity is increased at room temperature compared to -20^{0}C or -70^{0}C. This is illustrated by a comparison of results with samples collected specifically for measles and rubella RT-PCR with those first screened for virus antibody for surveillance, virus genome being detected in a considerably higher proportion of oral fluid samples collected specifically for RT-PCR. Further experiments need to be carried out to investigate more precisely the effect of cycles of freeze-thawing and loss of target RNA. This could involve "spiking" oral fluid samples with rubella virus and then subjecting them to a range of cycles of freeze-thawing before RT-PCR is carried out. Therefore, if oral fluid specimens are to be used for the detection of virus genome it is preferable to collect them specifically for this purpose followed by appropriate storage rather than referring to samples which have been previously subjected to other laboratory tests.

Analysis of results (Table 34) from samples originally collected for MMR surveillance within a week of the onset of symptoms showed the percentage of oral fluid samples in which genome was detected was highest for mumps (68.7%), followed by measles (53.8%) with the lowest percentage for rubella (41.7%). The most likely explanation why the percentage of oral fluid samples collected 0-7 days after onset of symptoms in which virus genome was detected was highest for mumps virus is that viral load in the oral cavity is highest for mumps. This is suggested by the characteristic swelling of the salivary glands with mumps infection and that mumps virus is also actively shed in oral fluid for up to 6 days before the onset of clinical disease (194). Other considerations include the comparative stability of the three viruses and the sensitivity of the three PCR detection assays.

Analysis of results from samples collected more than 7 days after onset of symptoms (Table 34) shows the percentage of oral fluid samples in which genome was detected decreased for each virus, though the proportion in which measles virus genome was detected was considerably higher than for mumps and rubella, especially when considering those samples collected >14 days after onset of symptoms. The proportion in which rubella virus genome was detected was particularly low for those samples collected >7 days after the onset of symptoms. Logistic regression analysis showed the difference in the detection of measles genome compared to mumps or rubella with time after onset of symptoms was significant ($p < 0.001$).

In attempting to explain these observations the incubation period of each virus infection must be considered spanning the time from exposure to the appearance of disease. All samples collected for this study were obtained after the onset of

symptoms. The incubation period of measles is 10-14 days, ~18 days for mumps and 16-21 days for rubella (67, 194, 195). Therefore once symptoms become evident, the immune system will have had greater opportunity to react and clear mumps and rubella virus in comparison to measles virus, particularly for those samples collected >7 days after onset of symptoms. Once symptoms of rubella become evident the entire clinical syndrome clears rapidly over several days (195) which is consistent with the low proportion of samples collected >7 days after the onset of symptoms in which rubella genome was detected. It may also be possible that a longer lasting viraemia occurs with measles than mumps or rubella, and that the immune system may take longer to clear measles virus.

Finally, the differences in terms of the proportion in which each virus genome could be detected as collection time increased after the onset of symptoms, may reflect protection afforded to the virus genome from degradative enzymes and the effects of freeze-thawing by the capsid and envelope. Results in this study suggest that the measles virus genome may be better protected in this respect than that of mumps and rubella. This may be determined by the extent to which enzymes which degrade nucleic acid are able to penetrate the capsid or how exposed or near the capsid surface the viral RNA is. Alternatively, viral RNA may form an important structural component of the capsid for those viruses where the genome is more easily degraded which may be the case for rubella (56).

In conclusion, this and other studies (29, 80) have shown that oral fluid can be used for the detection of measles, mumps and rubella virus genome and subsequently be useful for both the diagnosis and the molecular epidemiology of each of these virus

infections. This represents an important new application for the use of oral fluids in infectious disease testing. Apart from a few studies in which HBsAg was detected in saliva (21, 26, 146, 158), oral fluids have mainly been used for the detection of virus specific antibody (82, 119, 120, 139-141, 144, 157, 186, 187). The work described here shows that oral fluid can also be used to detect and characterise virus genomes.

It is of practical significance that oral fluid specimens should be collected soon after the onset of clinical symptoms, ideally within a week, stored at -70^{0}C and subjected to as few cycles of freeze-thawing as possible prior to RT-PCR assay. Measles, mumps and rubella virus genome was detected in oral fluid samples collected more than 14 days after onset of symptoms, though the likelihood of doing so was greatly reduced, particularly for rubella and mumps. Oral fluid samples are a safe and practical alternative to other types of clinical specimen for detecting measles, mumps and rubella virus genome. Coupled to the compliance advantages for collector and convenience to the patient, this procedure has potential to gather much interesting data on the genetic variation of viruses.

CONCLUSIONS

The work described in this part of the study focused on the development of a RT-PCR assay for rubella which enabled the molecular epidemiology of the virus to be investigated. A knowledge of molecular epidemiology can be used:

- to investigate transmission patterns
- to assess and measure the effectiveness of control strategies
- to link or unlink outbreaks and assist in the analysis of severe or unusual cases

This information augments that provided by serological studies to give a more complete understanding of the epidemiology of important vaccine preventable infectious diseases.

The RT-PCR assay developed was designed to be useful for both clinical diagnosis and the molecular epidemiology of rubella virus. This enabled the assay to be used to assist in the rapid diagnosis of suspected cases of congenital rubella syndrome. To date there have been few studies investigating the molecular epidemiology of rubella. Whilst the current understanding of the molecular epidemiology of rubella is far from complete, the application of the RT-PCR to the genetic characterisation of rubella virus strains in this study has contributed to the understanding of how the virus varies geographically, in identifying strains of the virus recently circulating in Europe and in demonstrating that it is possible to classify RV further than has previously been described.

The final part of this study focused on technical aspects of using oral fluid samples for molecular studies. It was demonstrated that in addition to being used for antibody

detection, oral fluid is also a viable clinical specimen to use for the detection and amplification of virus genome and particularly useful for early diagnosis of measles and mumps as well as rubella virus infections. The timing of sample collection and subsequent storage was shown to be critical. For optimal PCR results samples should be collected within 14 days of the onset of symptoms and stored at least at -20^{0}C prior to testing. The use of samples for other laboratory purposes (such as antibody detection) and subsequent cycles of freeze thawing prior to RT-PCR was associated with a decreased rate of detecting virus genome, though further work is required to investigate this in detail.

APPENDIX 3

3.1 Nucleic acid extraction and RT-PCR for cDNA synthesis

3.1a "Boom extraction" (silica method)

- 860ul L6 (Severn Biotech Ltd, Cat. No. 20-8600) and 40ul silica (Severn Biotech Ltd, Cat. No. 20-8000) are added to 100ul specimen and the mixture vortexed for 10 seconds and kept at room temperature for 10 minutes (mix or vortex every 2-3 minutes).
- The mixture is centrifuged for 15 seconds and the supernatant discarded.
- The silica pellet is washed twice with L2 (Severn Biotech Ltd, Cat. No. 20-8200), twice with 70% ethanol and once with 100% acetone (1ml wash solution, vortex briefly, centrifuge for 15 seconds and discard supernatant).
- The silica pellet is dried at 56^{0}C with an open lid for 10 minutes.
- Add 59ul of Nuclease-free water (Promega) and 1ul of RNAsin (Promega), vortex, and incubate for 10 minutes at 56^{0}C and overnight at 4^{0}C.
- Centrifuge for 2 minutes and take 40ul of the supernatant for RT-PCR for cDNA synthesis.

3.1b RT-PCR for cDNA synthesis

- 60ul of the following reaction mixture is added to 40ul of extracted RNA:

REAGENT	**VOLUME**
10x PCR buffer*	10ul
50mM $MgCl_2$*	3ul
10mM dNTPs*	2ul
Reverse Transcriptase (200U/ul)*	2ul
RNAsin (40U/ul)**	1ul
pd(N6) ["random primers"]*	1ul
Nuclease-free water**	41ul
TOTAL	**60ul**

*GIBCO BRL, ** Promega (Promega Corporation 608-274-4330 Madison, WI 53711-5399 USA)

- The reaction mixture is incubated at room temperature for 10 minutes, at 37°C for 1 hour, at 95°C for 5 minutes and cooled on ice for 3 minutes.
- The synthesized cDNA is then stored at -20°C or used for PCR.

3.2 TOPO TA Cloning Kit

3.2a Preparation

- For each transformation one vial of competent cells and one selective plate is needed.
- Equilibrate a water bath to 42°C.
- Thaw the vial of SOC medium from Box 2 and bring to room temperature.
- Warm LB plates containing 50 ug/ml ampicillin OR 50 ug/ml kanamycin at 37°C for 30 minutes.
- Spread 40 ul of 40 mg/ml X-gal on each LB plate and incubate at 37°C until ready for use.
- Thaw on ice 1 vial of One Shot™ cells for each transformation. Place the B-mercapto-ethanol on ice.

3.2b TOPO-Cloning Reaction

- In general, 0.5 to 2 ul of a typical PCR sample (10 ng/ul) with an average insert length of 400 to 1000 bp will give the proper insert:vector ratio for TOPO-Cloning.
- Set up the following 5 ul TOPO-Cloning reaction:

Fresh PCR product	0.5 to 2 ul
Sterile Water	add to a final volume of 4 ul
pCR-TOPO vector	1 ul
Final Volume	5 ul

- Mix gently and incubate for 5 minutes at room temperature (~25°C). For the best possible results, do not leave for more than 5 minutes or the transformation and cloning efficiencies will decrease.
- Briefly centrifuge and place tube on ice. Proceed immediately to One Shot Transformation Reaction, below.

3.2c One Shot Transformation Reaction

- Add 2 ul of 0.5 M B-mercaptoethanol to each vial of competent cells and mix by stirring gently with the pipette tip. ***DO NOT MIX BY PIPETTING UP AND DOWN.***
- Add 2 ul of the TOPO-Cloning reaction into a vial of One Shot cells and mix gently.
- Incubate on ice for 30 minutes.
- Heat shock the cells for 30 seconds at 42°C without shaking.
- Immediately transfer the tubes to ice and incubate for 2 minutes.
- Add 250 ul of room temperature SOC medium.
- Cap the tube tightly and shake the tube horizontally at 37°C for 30 minutes (ampicillin selection) or 1 hour (kanamycin selection). Place on ice.
- Spread 50-100ul from each transformation on a prewarmed plate and incubate overnight at 37°C.
- An efficient TOPO-Cloning reaction will produce hundreds of colonies. Pick ~10 white or light blue colonies for analysis.

3.2d Analysis of positive clones (PCR method)

- Prepare a PCR cocktail consisting of PCR buffer, dNTPs, appropriate primers, *Taq* polymerase and RNAse free water. Use 50 ul reaction volumes.
- Pick 10 colonies and resuspend them individually in 50 ul of the PCR cocktail.
- Incubate the reaction for 10 minutes at 94^{0}C to lyse the cells and inactivate nucleases.
- Amplify as appropriate for the PCR reaction being used.
- Visualise by agarose gel electrophoresis.

3.3 The GENECLEAN II Kit BIO 101 Inc.

3.3a Excision of DNA band

- The DNA band is excised from the ethidium bromide-stained agarose gel with a scalpel/razor blade.
- Long-wave UV light is used for a short a time as practical to minimise damage to the DNA sample.
- The approximate volume of the gel slice is determined (1gm = ~ 1ml) and transferred to a plastic tube (DO NOT use glass as the DNA will bind to it).

3.3b Add 3 volumes of NaI stock solution

- The volume of NaI added to a gel piece that contains DNA is approximately 3 times the volume of the solution (or weight of gel slice). For agarose with TBE add 1/2 volume of TBE Modifier and 4.5 volumes of NaI to a given volume of agarose.
- This keeps the final concentration of NaI above 4 molar.
- The tube is incubated at 55^{0}C for 2 minutes, mixed, and incubated again at 55^{0}C for 5 minutes until the agarose gel has completely dissociated.

3.3c Add GLASSMILK

- Vortex the insoluble silica matrix stock until all the contents are in suspension.
- Add 5ul of GLASSMILK suspension to solutions containing 5ug or less of DNA. Add an additional 1ul for each 0.5ug of DNA above 5ug.
- Mix well and incubate on ice for 5 minutes to allow binding of the DNA to the silica matrix.

3.3d Pellet the silica matrix with the bound DNA

- Spin the suspension in a microcentrifuge for 10 seconds.
- Discard the supernatant.

3.3e Wash pellet three times with NEW WASH

- 1ml of ice cold NEW WASH is added to the pellet.
- The pellet is resuspended.
- The suspension is spun in a microcentrifuge for 10 seconds and the supernatant discarded.
- This procedure is repeated twice.

3.3e Elution of DNA from GLASSMILK

- The washed pellet is resuspended in 20ul RNAse free water.
- The tube is incubated at 55^{0}C for 2 minutes and centrifuged for 30 seconds.
- The supernatant containing the eluted DNA is carefully removed and stored in a new tube. It can either be used immediately or stored at -30^{0}C until required.
- The presence of DNA the correct size can be checked by running 2ul of eluted product on a 2% agarose gel.

3.4 ABI Prism Dye terminator cycle sequencing ready reaction kit

- Prepare two sequencing PCR tubes for each purified PCR amplicon DNA sample.

- To the first tube add 8ul of ABI Prism Dye terminator mix, 10ul of the first DNA sample and 1.5ul of 5'second round primer (at 3.2pmol/ul). To the second tube add 8ul of ABI Prism Dye terminator mix, 10ul of the first DNA sample and 1.5ul of 3'second round primer (at 3.2pmol/ul). Repeat this process for subsequent samples. Therefore each DNA sample has a 5' strand tube and a 3' strand tube.
- Briefly centrifuge.
- Place the tubes in a thermocycler with a heated lid and run a sequencing programme.
- Add 50ul 95% ethanol and 2ul 3M NaOAc to each tube and incubate on ice for 10 minutes.
- Centrifuge at 13,500 rpm [17,000xg] (IEC Micromax) for 30 minutes.
- Remove the supernatant with a fine tip pastette.
- Add 200ul of 70% ethanol.
- Centrifuge at 13,500 rpm [17,000xg] (IEC Micromax) for 5 minutes.
- Remove the supernatant with a fine tip pastette.
- Leave the tubes to dry with an open lid at room temperature.
- Store the tubes at -20^{0}C until ready to send for sequencing.

3.5 Opti-Prime PCR Optimization Kit (Stratagene Cloning Systems, 1011 North Torrey Pines Road, La Jolla, CA 92037)

3.5a Optimal Buffer Determination

- Label 12 sterile microcentrifuge tubes with the numbers 1 through 12. Add 5ul of each of the 12 Opti-Prime 10x buffers to its corresponding microcentrifuge tube.
- In a separate microcentrifuge tube (on ice) make up an appropriate volume PCR mix containing MgCl2, dNTPs, primers, *Taq* polymerase and Nuclease-free water for the PCR reaction being optimised.
- Add 25ul of the PCR reaction mix to each of the 12 microcentrifuge tubes and add one drop of mineral oil to seal the reactions.
- Add 20ul of positive control to each of the 12 microcentrifuge tubes.
- Place the microcentrifuge tubes in a thermocycler and run the appropriate programme.
- If the PCR being optimised is a nested reaction, repeat the above steps adding 35ul of appropriate PCR reaction mix to each of the 12 microcentrifuge tubes with 10ul of first round product. Add one drop of mineral oil to seal the reactions. Place the microcentrifuge tubes in a thermocycler and run the appropriate programme.
- Load 10ul of each of the 12 final PCR products onto a 2% agarose gel. Electrophorese, stain with ethidium bromide and evaluate the PCR products for their correct size, for the desired PCR product yield and for the amount of non-specific background amplification products present.
- Determine the best buffer(s) and further optimise by the addition of adjuncts if necessary.

3.5b Optimal Adjunct Determination

- Select the appropriate 10x buffer and prepare a reaction mixture of all the components for the PCR reaction (excluding the adjuncts, which are added separately).
- Label seven sterile microcentrifuge tubes with the numbers 1 through 7. Add the adjuncts to each of the seven microcentrifuge tubes as indicated in the table below:

Tube No.	Adjunct	Final Concentration
1	5.0ul of formamide	5%
2	2.5ul of DMSO	5%
3	7.5ul of glycerol	15%
4	1.0ul of 750mM $(NH_4)_2SO_4$	15mM
5	3.0ul of 1.5mg/ml BSA	100ug/ml
6	0.5U of Perfect match DNA polymerase enhancer (genomic templates)	0.5U
7	5.0ul of sterile dH_2O	Control

- 30ul PCR reaction mix and 20ul positive control is added to each of the seven microcentrifuge tubes with a drop of mineral oil to seal the reactions.
- Place the microcentrifuge tubes in a thermocycler and run the appropriate programme.
- If the PCR being optimised is a nested reaction, repeat the above steps adding 35ul of appropriate PCR reaction mix to each of the 7 microcentrifuge tubes with 10ul of first round product. Add one drop of mineral oil to seal the reactions. Place the microcentrifuge tubes in a thermocycler and run the appropriate programme.
- Load 10ul of each of the 7 final PCR products onto a 2% agarose gel. Electrophorese, stain with ethidium bromide and evaluate the PCR products for their correct size, for the desired PCR product yield and for the amount of non-specific background amplification products present.

3.6 Measles RT-PCR (81)

This assay is designed to detect measles virus RNA in a variety of clinical specimens using primer pairs in the nucleocapsid (N) and matrix (M) genes in one reaction (dual target-PCR).

- The first round of PCR mixture contained 20ul of cDNA, 5 pmol of each primer (Mn1, Mn2R, Mm1 and Mm2R), 1.0U of *Taq*polymerase (Life Technologies, UK), 3ul of 10x PCR buffer (Bioline, UK) and made up to 50ul with sterile water (no dNTPs were added as they were already present from RT reaction).
- PCR mixture was then overlaid with one drop of light mineral oil.
- An initial denaturation step for 2 min at 95°C was followed by 25 cycles of 1 min at 94°C, 1 min at 50°C and 1 min at 72°C.
- For nested PCR, 3 ul of first round PCR product was added to 25 pmol of each primer (Mn3, Mn4R, Mm3 and Mm4R), 200mM of each dNTP and 1.0U of *Taq*polymerase, 5ul of 10x PCR buffer and made up to 50ul with sterile water.
- The reactions were processed for 25 cycles of 1 min at 94°C, 1 min at 55°C and 1 in at 72°C after an initial denaturation for 2 min at 95°C.
- The two differently sized PCR products were produced in a single PCR mixture and detected by electrophoresis in a 3% agarose gel.
- The primers for the M gene gave an amplification product of 187 base pairs and those for the N gene gave a product of 317 base pairs.

3.7 Mumps RT-PCR (79)

- 20ul of cDNA was added to the first round PCR, and 10ul (2ul for positive control) of the first-round PCR amplicon was then used as a template for the nested PCR.

- Nested PCR amplification of a 639-bp fragment encompassing the entire small hydrophobic (SH) gene was done with 2 sets of primers.
- Primers SH1 and SH2R, each at a concentration of 10 pmol, were used for the first-round PCR, and SH3 and SH4R were used at a concentration of 25 pmol for the nested PCR.
- The PCR amplification was done as follows: an initial incubation for 2 min at 95^0C was followed 25 cycles of 1 min at 95^0C, 1.5 min at either 50^0C (for the first round) or 55^0C (for the nested PCR), 2 min at 720C, and a final extension step for 5 min at 72^0C.
- Tissue culture fluid of a mumps virus isolate (the Taylor strain [Tay/UK50s]) was used as a positive control.

SUMMARY

This study was designed to investigate the extent to which oral fluid can be used to study the epidemiology of vaccine preventable virus infections, taking rubella and hepatitis B as examples. This involved the measurement of virus specific IgG antibody and the detection of virus genome in oral fluid to investigate both the seroprevalence and the molecular epidemiology of infection by these two viruses. The major advantage of using oral fluid for these purposes is based around the accessibility and compliance advantages of obtaining oral fluid samples in comparison to more invasive types of clinical specimens that can be used.

Assays which measure specific IgG may be used in two ways:

- fordetecting evidence of past infection in the individual
- the determining levels of immunity in a population

The performance characteristics of the assay will determine for which purpose it is best suited. Assays which demonstrate a particularly high level of sensitivity and specificity in comparison to a recognised gold standard may be used for testing in the individual. Those whose performance characteristics are not so good may none the less still be usefully employed in population immunity testing where correct diagnosis on an individual level is not critical. The availability and performance characteristics of suitable serological assays which utilise body fluids more accessible than blood - such as oral fluid - make them particularly useful for the studies of immunity in populations and for the surveillance and laboratory confirmation of clinically diagnosed cases.

The detection, amplification and subsequent nucleotide sequencing of virus genome present in a sample enables strain variation to be studied and chains of transmission to be investigated. This information is particularly useful when effective vaccination programmes are in place and clinical cases identified occur mainly as a result of importations of infection aquired abroad.

The work described in Chapter 1 addressed the important practical issue of how to collect oral fluid samples. Three types of oral fluid collection devices were compared to identify the most suitable in terms of the quality of oral fluid collected and its suitability for specific IgG antibody screening. Two populations in which an understanding of the epidemiology of vaccine preventable virus infections is important were used to provide samples. These were a rural African population where vaccination coverage is low or absent and limited to certain infections only and a population of young children from the UK, a developed country where an extensive vaccination schedule is in place. This work showed that all three of the oral fluid collection devices yielded a sample that could be used for the ***qualitative*** determination of virus specific IgG. However, the Oracol yielded oral fluid of the highest quality in terms of the total and specific antibody concentration and was the most acceptable device to participants from both populations in the study. The Oracol was also considerably cheaper than the other two makes of device. Therefore the Oracol was identified as the oral fluid collection device of choice.

A comparison of the total IgG concentration in samples from the two study populations was made and was shown to be approximately five times higher in those subjects from Ethiopia. This may reflect the developmental stage or maturity of the

immune system of the participants involved as the Ethiopian population covered a broad age spectrum whilst the children from north Hertfordshire were all aged between 3.5 and 5 years. Alternatively, susceptibility to infectious disease and level of humoral response may be genetically determined and therefore vary between ethnic groups.

The second chapter dealt with the development of simple robust ELISA assays that could be used to measure virus specific IgG antibody in oral fluid samples. The assays developed were designed to replace existing radioimmunoassays and needed to be suitable for use in basic laboratories in developing countries. There is currently no vaccination programme in place in rural Africa for rubella and only some African countries (e.g. Gambia, South Africa) have a programme of vaccination for hepatitis B, the virus infections chosen for this study. Suitable ELISA tests capable of detecting specific IgG in oral fluid against both these infections would provide a means to investigate the epidemiology of rubella and hepatitis B in populations from rural Africa. By utilising the compliance advantages of oral fluid such tests would assist in the design of suitable vaccination strategies and monitor their effectiveness.

The rubella GACELISA developed used the fluorescein isothiocyanate (FITC)-anti-FITC amplification system and was shown to be more sensitive than radioimmunoassay and, in comparison to a commercial serum ELISA, performed well using samples from pediatric populations. However, sensitivity was shown to decrease with age. The anti-HBc GACELISA was developed using a conventional detection system comprising of an anti-mouse HRPO conjugated antibody due to the lack of a suitable volume of anti-HBc monoclonal antibody for FITC conjugation. The

overall sensitivity of the resulting assay was poor in comparison to a commercial serum ELISA and was also shown to decrease with age. Overall, the rubella GACELISA was most suitable for population immunity testing rather than for diagnosis in the individual, though did perform well with samples from paediatric populations. In contrast, the performance characteristics of the anti-HBc GACELISA were not high enough for it to be used for either purpose.

Subsequent development and use of a measles FITC/anti-FITC GACELISA suitable for use with oral fluid developed by Nigatu et al (126) showed it compared well with serum ELISA and performed well in all age groups. These results suggested that the performance of virus specific IgG capture ELISAs designed for use with oral fluid may be dependant on the biological characteristics of the particular virus infection and the humoral response directed against it as well as the quality of reagents available.

Future work on the development of assays designed to detect specific antibody in oral fluid should focus on three main areas:

- Improving assay sensitivity. This is likely to involve the development of new detection systems that are able to accurately detect the low levels of virus specific antibody found particularly in oral fluid from older age groups, a major limiting factor of the GACELISAs for rubella and hepatitis B.
- The conversion and subsequent optimisation of the current ELISA assays that detect specific IgG in oral fluid to those that detect ***virus specific IgM*** in oral fluid. This will enable the laboratory diagnosis and confirmation of important vaccine preventable infections to be made in basic laboratories in developing countries.

- The development of further ELISA assays for the detection of specific IgG in oral fluid against other important infections, including parasites and bacteria as well as other viruses, that will be useful in both the developed and developing world.

The work described in chapter 3 investigated the molecular epidemiology of rubella and studied the extent to which oral fluid samples may be used for the detection and characterisation of the virus genome.

The extent to which oral fluid samples may be used for the detection of measles, mumps and rubella genome was investigated with regard to **(a)** collection period after the onset of symptoms, **(b)** storage and **(c)** prior use of samples. Results showed that virus genome for all three infections was detectable in oral fluid samples up to 14 days after the onset of symptoms. However the detection of virus genome was greatly increased if samples were collected specifically for RT-PCR and stored at -30^{o}C or below prior to use. Oral fluid samples are therefore a safe and practical clinical specimen for molecular studies involving measles, mumps and rubella. They are useful for early diagnosis since specific IgM may not be detectable for a number of days after the onset of symptoms.

A nested RT-PCR assay was developed that targeted a region of the E1 gene of the rubella genome and generated a second round amplicon of suitable length for both diagnosis and phylogenic analysis. This was then used on a variety of clinical specimens, including oral fluid, collected from Europe, Asia and South America. The assay was also shown to have potential for the diagnosis of congenital rubella.

Genotyping demonstrated that rubella can be classified further than has previously been described and could be used to monitor circulating strains.

Future studies on rubella PCR and genotyping should focus on:

- the characterisation of rubella from parts of the globe as yet unsampled (e.g. Africa) and under represented (e.g. India, South America) and the development of a more extensive typing scheme. This may then enable the development of a probe system which could be used for rapid and more cost effective detection and genotyping of rubella.
- sequencing the complete rubella genome and the development of an RT-PCR that targets other regions of the genome which may be suitable for diagnosis and molecular epidemiology of rubella.
- the extent to which other more appropriate clinical specimens, such as amniotic fluid, may be used in the RT-PCR assay for the diagnosis of congenital rubella.
- the development of a real time RT-PCR assay that uses high speed thermal cycling (e.g. LightCycler). This would be very useful for the rapid investigation of suspected cases of congenital rubella.

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